

ACTA MICROBIOLOGICA

ACADEMIAE SCIENTIARUM
HUNGARICAE

ADIUVANTIBUS

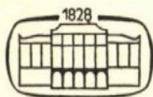
L. ALFÖLDI, I. BÉLÁDI, I. DÖMÖK, E. FARKAS, G. FARKAS,
L. FERENCZY, I. FÖLDES, I. HORVÁTH, I. JOÓ, I. KÉTYI,
L. KESZTYŰS, B. LÁNYI, I. NÁSZ, I. M. SZABÓ, L. VÁCZI,
J. WEISZFEILER

REDIGIT

G. IVÁNOVICS

TOMUS XXIV

FASCICULUS I



AKADÉMIAI KIADÓ, BUDAPEST

1977

AMAHA 5 24(1) 1-106 1977

Acta microbiol. Acad. Sci. hung.

ACTA MICROBIOLOGICA

A MAGYAR TUDOMÁNYOS AKADÉMIA
MIKROBIOLÓGIAI KÖZLEMÉNYEI

KIADÓHIVATAL: 1954 BUDAPEST, ALKOTMÁNY UTCA 21.

A Szerkesztőbizottság elnöke :
IVÁNOVICS GYÖRGY
akadémikus

Főszerkesztő :
LÁNYI BÉLA

Az *Acta Microbiologica* német, angol, francia és orosz nyelven közöl értekezéseket a mikrobiológia tárgyköréből.

Az *Acta Microbiologica* változó terjedelmű füzetekben jelenik meg. Több füzet alkot egy kötetet.

A közlésre szánt kéziratok a következő címre küldendőek:

Acta Microbiologica, Országos Közegészségügyi Intézet, 1966 Budapest, Pf. 64.

Ugyanerre a címre küldendő minden szerkesztőségi levelezés.

Megrendelhető a belföld számára az Akadémiai Kiadónál (1363 Budapest Pf. 24 Bankszámla 215-11448), a külföld számára pedig a „Kultúra” Külkereskedelmi Vállalatnál (1389 Budapest 62, Pf. 149, Bankszámla: 218-10990) vagy annak külföldi képviselőiteinél és bizományosainál.

The *Acta Microbiologica* publish papers on microbiological subjects in English, German, French and Russian.

The *Acta Microbiologica* appear in parts of varying size, making up volumes.

Manuscripts should be addressed to

Acta Microbiologica, National Institute of Hygiene, H-1966 Budapest, P. O. B. 64, Hungary

Correspondence with the editors should be sent to the same address.

The rate of subscription is \$ 32.00 a volume.

Orders may be placed with “Kultúra” Foreign Trade Company (H-1389 Budapest 62, P. O. B. 149, Account No. 218-10990) or with representatives abroad.

ACTA MICROBIOLOGICA

ACADEMIAE SCIENTIARUM
HUNGARICAE

ADIUVANTIBUS

L. ALFÖLDI, I. BÉLÁDI, I. DÖMÖK, E. FARKAS, G. FARKAS,
L. FERENCZY, I. FÖLDES, I. HORVÁTH, I. JOÓ, I. KÉTYI,
L. KESZTYŰS, B. LÁNYI, I. NÁSZ, I. M. SZABÓ, L. VÁCZI,
J. WEISZFEILER

REDIGIT

G. IVÁNOVICS

TOMUS XXIV



AKADÉMIAI KIADÓ, BUDAPEST

1977

Acta microbiol. Acad. Sci. hung.



ACTA MICROBIOLOGICA

TOMUS XXIV

INDEX

Fasciculus 1

<i>Molnár, J., Mándi, Y., Holland, I. B., Schneider, Gy.</i> : Antibacterial Effect, Plasmid Curing Activity and Chemical Structure of Some Tricyclic Compounds	1
<i>Dhikidze, E. K., Kavtaradze, K. N., Krilova, R. I., Rauss, K., Kétyi, I., Vertényi, A.</i> : Oral Immunization of Monkeys with Polyvalent Dysentery Vaccine	7
<i>Gergely, L., Czeglédi, J., Szalka, A., Váczi, L., Binder, L.</i> : Epstein-Barr Virus and Cytomegalovirus Antibodies in Infectious Mononucleosis	13
<i>Boldogh, I., Gönczöl, É., Váczi, L.</i> : Cytomegalovirus-Induced Membrane Antigens in Productively Infected Cells	21
<i>Sipka, S., Boldogh, I., Szilágyi, T.</i> : Macrophage Disappearance Reaction in <i>Rana esculenta</i> Induced by Specific Antigen and Rabbit Lymphokines	29
<i>Singh, A., Mohan, K. S., Sethi, S. K., Vadehra, D. V.</i> : Cross-Neutralization Reactions of <i>Escherichia coli</i> and <i>Vibrio cholerae</i> Enterotoxins as Studied by Rabbit Skin Inoculation Test	33
<i>Seventh Congress of the Hungarian Society of Microbiology, Budapest, September 6—9, 1976.</i>	
Abstracts of papers	41
Plenary Session	43
Immunology	48
Interaction of pesticides and microorganisms	57
Virology	63
Bacterial genetics	80
Bacteriology	92
Mycology	101

Fasciculus 2

<i>Lantos, J.</i> : Transfer of Erythromycin Resistance in <i>Staphylococcus aureus</i>	107
<i>Czirók, É., Milch, H., Madár, J., Semjén, G.</i> : Characterization of <i>Escherichia coli</i> Serogroups Causing Meningitis, Sepsis and Enteritis. I. Serological Properties and Animal Pathogenicity of O18, O78 and O83 Isolates	115
<i>Milch, H., Czirók, É., Madár, J., Semjén, G.</i> : Characterization of <i>Escherichia coli</i> Serogroups Causing Meningitis, Sepsis and Enteritis. II. Classification of <i>Escherichia coli</i> O78 Strains by Phage Sensitivity, Colicin Type and Antibiotic Resistance	127
<i>Financsek, I., Kétyi, I.</i> : Phenotypic Correction of Nonsense Mutation Carrying Non-Converting PE5 Phages in <i>Shigella flexneri</i> with Suppressor Gene	139
<i>Szilágyi, T., Deseő, Gy.</i> : Distribution of ³² P-Labelled Endotoxin in the Frog	149
Abstracts of 1975 European Immunology Meeting. 16th Workshop „Bacterial Endotoxins”	157

Fasciculus 3

Karaszeva, V., Sziklai, I., Ördögh, M.: Quantitation of Seven Elements in <i>Mycobacterium phlei</i> and <i>Mycobacterium bovis</i> by Neutron Activation Analysis	175
Ádám, É., Nász, I., Medveczky, P.: Comparative Studies on the Soluble Proteins of Adenovirus Type 1	181
Nagy, G., Mezey, I.: The Use of Ion Exchange Chromatography for Demonstration of Rubella-Specific IgM Antibodies	189
Cameron, J., Stainer, D. W.: Pertussis Vaccine — Fact and Fiction	195
Bergmann, P., Mebel, S., Rustenbach, S.: Morphology of <i>Salmonella typhi</i> in Different Inactivation Techniques	207
Polotsky, Y. E., Dragunskaya, E. M., Seliverstova, V. G., Avdeeva, T. A., Chakhutinskaya, M. G., Kétyi, I., Vertényi, A., Ralovich, B., Emödy, L., Málovics, I., Safonova, N. V., Snigirevskaya, E. S., Karyagina, E. I.: Pathogenic Effect of Enterotoxigenic <i>Escherichia coli</i> and <i>Escherichia coli</i> Causing Infantile Diarrhoea	221
Szabó, I. M., Kondics, L., Marton, M., Buti, I.: Changes in the Surface Layer (Sheath) of the Cell Wall of Streptomycetes During Sporulation	237
Szilágyi, T., Tóth, S., Lévai, G.: Influence of Hypothermia on the Generalized Shwartzman Reaction	247
Rische, H., Tschäpe, H., Witte, W.: Surveillance of R-Plasmids	253
M. Ádám, M., Kovách, C., Kerekes, L., Béry, R.: Note. Indole Positive Variant of <i>Shigella boydii</i> 1	261

Fasciculus 4

Kuryłowicz, W.: The Site of Antibiotic Accumulation in Streptomycetes and <i>Penicillium chrysogenum</i>	263
Nyerges, G., Jaszovszky, I., Pintér, A., Hompó, Zs.: Investigations into the Encephalitogenic Activity of the Hempt Vaccine in Guinea Pigs	273
Khavkin, T. N., Freidlin, I. S., Sakharova, I. Ya., Ioffe, V. A.: Vital Fluorescence Microscopy of Lysosomes in Cultured Mouse Peritoneal Macrophages During their Interactions with Microorganisms and Active Substances. I. Vital Staining and Fluorescence Microscopy of Macrophages	281
Freidlin, I. S., Khavkin, T. N., Artemenko, N. K., Sakharova, I. Ya.: Vital Fluorescence Microscopy of Lysosomes in Cultured Mouse Peritoneal Macrophages During their Interactions with Microorganisms and Active Substances. II. Interactions of Macrophages with a Non-Pathogenic Strain of <i>Escherichia coli</i>	293
Budai, J., György, B., Szécsény, Gy., Berta, K.: Effect on Plants of Sera from Patients with Hepatitis	303
Simon, M.: Exclusion of False Positive Reactions Due to IgG-Binding Fc-Receptor(s) in Herpesvirus Serology	307
Nagy, G., Mezey, I., Molnár, E.: Simple Ion Exchange Batch Technique for Detection of IgM Antibodies Against Rubella and Flavivirus Antigens	317
Tálas, M., Zakharova, L. G., Stöger, I., Altstein, A. D., Piker, E. G.: Quantitative Analysis of MC29 Virus in Plasma of Hepatoma-Bearing Turkey Poults by Determination of Reverse Transcriptase Activity and Radioimmuno-Assay	323
D. Tóth, F., Jakó, J., Váczi, L., Rák, K., Rédei, I.: Immunological Demonstration of Oncornavirus Genome in Leucocytes in Acute and Chronic Myelogenous Leukaemia and Preleukaemia	331
Hollós, I., Pálfi, Á., György, B., Berta, K.: Preliminary Report. Experiments Concerning the Specificity and Mechanism of Action of HB _s Ag in <i>Nicotiana glauca</i>	341
Books Received	345

ANTIBACTERIAL EFFECT, PLASMID CURING ACTIVITY AND CHEMICAL STRUCTURE OF SOME TRICYCLIC COMPOUNDS

J. MOLNÁR, YVETTE MÁNDI, I. B. HOLLAND and GY. SCHNEIDER

*Institute of Microbiology, University Medical School, Szeged, Hungary, Department of Genetics,
School of Biological Sciences, University of Leicester, Leicester, England and Institute of Organic
Chemistry, University of Szeged, Szeged, Hungary*

(Received February 12, 1976)

Diethazine, amitriptyline and imipramine showed bacteriostatic and bactericidal effect on different bacteria. Chlorpromazine sulphoxide and fluorescein were ineffective even at 1000 µg/ml. The antibacterial compounds deleted at 40-70% frequency the F⁺lac plasmid of *Escherichia coli* K12 LE-140.

As shown earlier, certain phenothiazine compounds inhibit the growth of different bacteria. The substances exerted a plasmid curing effect on R factor of multiple resistant *Escherichia coli* [1, 2] and on F⁺lac factor of *E. coli* at concentrations not influencing markedly the multiplication of bacteria [3].

In the present study we examined whether the antibacterial and plasmid curing activities of phenothiazine derivatives similar in planar molecular structure to acridine orange were associated with the presence of two heteroatoms (S, N) in the middle ring. It has been supposed that by modifying the tricyclic molecule, the structures responsible for the two kinds of activity may be separated. For this purpose three groups of tricyclic compounds were examined: (i) the phenothiazine derivatives diethazine and chlorpromazine sulphoxide containing two heteroatoms (S, N) in their middle ring; (ii) imipramine with one heteroatom (N) and fluorescein with O atom; (iii) amitriptyline not containing heteroatoms.

Materials and methods

Substances. Imipramine (Melipramin[®]), amitriptyline⁺ (Teperin[®]), diethazine (Apar-kazin[®]), E. Gy. T. Pharmaceutical Works, Budapest. Fluorescein. Chlorpromazine sulphoxide prepared by the method of FISHMAN and GOLDENBERG [4].

Bacterial strains. *Bacillus anthracis* VR, *Escherichia coli* K12 LE-140, *Streptococcus pyogenes*, *Streptococcus viridans*, *Staphylococcus aureus*, *Corynebacterium hofmannii*, *Streptococcus pneumoniae*, *Proteus vulgaris*, *Pseudomonas aeruginosa*. Except the first two strains, the bacteria were isolated from clinical specimens. Curing of F⁺lac plasmid was examined with strain *E. coli* K12 LE-140 (T⁺₈T⁺₁Sm^RLac⁻Su⁻λ^RMal⁻).

Culture media. MTE broth [5] was modified as follows: NH₄Cl, 1 g; K₂HPO₄, 7 g; NaH₂PO₄, 3 g; NaCl, 8 g; glucose, 1 g; Bacto Tryptone (Difco), 10 g; Bacto Yeast Extract (Difco), 1 g; distilled water, to 1000 ml. The pH was adjusted to 7.2 before autoclaving. Eosin methylene blue (EMB) agar was used for detecting lac⁻ colonies.

Antibacterial effect. An overnight preculture at aliquots of 0.05 ml was transferred to 5 ml amounts of MTE broth containing the compounds to be examined at different concentrations. After incubation at 37 °C for 24 hr, the optical densities were measured in the Bausch

and Lomb Spectronic 20 photometer at 620 nm. The lowest amount of the compound causing complete inhibition of growth was regarded as the minimum inhibitory concentration.

Bactericidal effect. MTE broth culture ($OD = 0.2$) was diluted at 2 ml portions and the compounds were added at different concentrations. The tubes were incubated in water bath at $37^\circ C$ for 120 min, then the number of colony formers was determined by plating onto MTE agar.

Curing of *F*⁺lac plasmid was examined as described earlier [3]. An overnight preculture of *E. coli* K12 LE-140 was diluted 10^{-4} and inoculated at 0.05 ml aliquots (approximately 5×10^3 cells) into 5.0 ml MTE broth. Then different concentrations (0–200 $\mu g/ml$) of the substances were added and the tubes were incubated at $37^\circ C$ for 24 hr. From tubes showing growth, different dilutions were prepared and plated at 0.1 ml amounts on EMB agar. The plates were incubated at $37^\circ C$ for 24 hr then counted for *lac*[−] (pink) and *lac*⁺ (deep violet) colonies.

Results

Antibacterial activity. Imipramine and amitriptyline exerted bacteriostatic activity at about the same concentration (80–120 $\mu g/ml$ for Gram-positive and 160–230 $\mu g/ml$ for Gram-negative organisms). Diethazine acted more markedly on Gram-positive (40–60 $\mu g/ml$) than on Gram-negative bacteria (160–180 $\mu g/ml$). *P. aeruginosa* was not inhibited even by 1000 $\mu g/ml$ concentrations of the substance (Table I).

Table I

Bacteriostatic effect of imipramine, amitriptyline and diethazine

Bacterial strains	Minimum bacteriostatic concentration, $\mu g/ml$		
	imipramine	amitriptyline	diethazine
<i>B. anthracis</i> VR	80	100	50
<i>C. hofmannii</i>	120	100	40
<i>S. pneumoniae</i>	100	80	40
<i>S. aureus</i>	120	110	50
<i>S. pyogenes</i>	110	110	50
<i>S. viridans</i>	120	100	60
<i>E. coli</i> K12 LE-140	180	160	160
<i>K. pneumoniae</i>	180	150	160
<i>P. vulgaris</i>	230	170	180
<i>P. aeruginosa</i>	—	—	—

Compounds showing bacteriostatic activity were examined with multiplying broth cultures of *E. coli* K12 LE-140. When added at 250 $\mu g/ml$ to $OD = 0.2$ cultures, all three compounds terminated growth (Fig. 1).

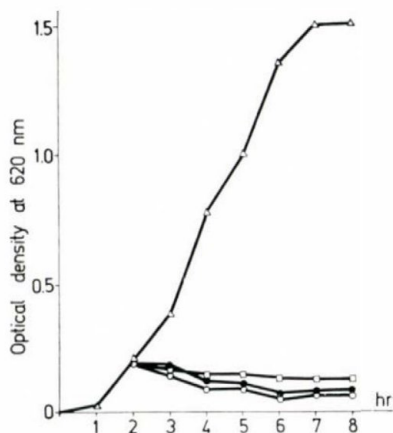


Fig. 1. Effect of imipramine (●—●), amitriptyline (○—○) and diethazine (□—□) on the growth of *E. coli* K12; control culture: △—△

Although the optical density of the cultures decreased somewhat, marked lysis could not be observed.

As shown in Fig. 2, varying with the concentration of the compound, the number of colony-formers decreased in the cultures. Diethazine and amitriptyline showed a more marked bactericidal activity than imipramine.

Plasmid curing activity. With subinhibitory concentrations of the five compounds the following results were obtained. Depending on the concentration, imipramine deleted F⁺lac plasmid at 0–41% frequency (Table II). The number of colony-formers gradually decreased with the increase of con-

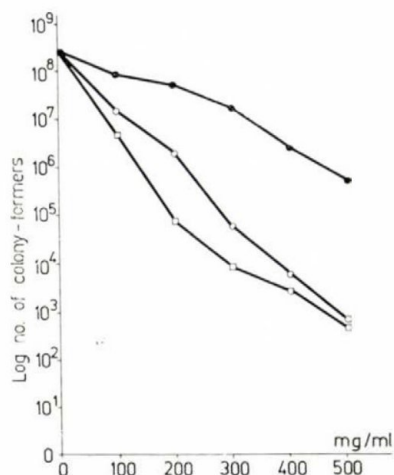


Fig. 2. Bactericidal effect of imipramine (●—●), amitriptyline (○—○) and diethazine (□—□) on multiplying cells of *E. coli* K12

centration. Amitriptyline exerted a maximum curing efficiency of 70% (Table III). Chlorpromazine sulphoxide and fluorescein up to 400 $\mu\text{g/ml}$ failed to show any curing effect. According to earlier experiments [3] diethazine (40 $\mu\text{g/ml}$) showed curing in the same system at 22% frequency.

Table II
F⁺lac-plasmid curing activity of imipramine

Imipramine, $\mu\text{g/ml}$	Number of viable cells	Number of colonies tested	Efficiency of curing, per cent
0	2×10^9	1274	0
40	2×10^9	800	0
50	1.5×10^9	1300	0.2
80	5×10^8	1000	0.2
100	9×10^7	500	5.0
120	2.5×10^7	650	8.0
150	3×10^7	2000	35–40
160	3×10^7	1063	41
200	no bacterial growth		

Table III
F⁺lac-plasmid curing activity of amitriptyline

Amitriptyline, $\mu\text{g/ml}$	Number of viable cells	Number of colonies tested	Efficiency of curing, per cent
0	10^9	1200	0
25	10^8	2000	0
50	9×10^7	850	5.0
60	10^8	2162	0
80	5×10^7	505	6.3
100	2×10^7	1250	50.0
150	1.2×10^7	900	70.0
200	no bacterial growth		

Discussion

The antibacterial effect of phenothiazine [6, 7] and its derivative chlorpromazine [1, 8, 9–12] has been investigated by several authors. Other phenothiazine compounds such as promethazine, levomepromazine and diethazine also exert antibacterial [2] and plasmid curing effect [1–3, 13].

In man, chlorpromazine inhibits monoamine oxidase, whereas chlorpromazine sulphoxide is ineffective on the enzyme [14]. In our experiments the latter exerted neither bacteriostatic nor plasmid curing activity. These data indicate that in phenothiazine derivatives, which are structurally related to acridine orange, the antibacterial and curing effect is masked by oxygen bound to the sulphur atom of the middle ring. Since the dimethylamino propyl group linked to the nitrogen atom of the middle ring is also present in the sulphoxide molecule, its role in antibacterial and plasmid curing activity can theoretically be excluded. Since fluorescein, containing one heteroatom (oxygen) was also ineffective, it seems that the lack of antibacterial and curing activity is associated with the oxygen atom present in the ring (fluorescein) or linked to the ring (chlorpromazine sulphoxide). In contrast, imipramine containing nitrogen as a single heteroatom and amitriptyline devoid of heteroatom, were effective. It would appear, accordingly, that the antibacterial and curing activity depends on the charge distribution of the tricyclic ring.

Proflavine and acridine orange analogue tricyclic compounds containing different heteroatoms in their middle ring, may be intercalated between the base pairs of DNA [15]. As the G–C pair is more polarized than the A–T pair, it interacts more readily with the slightly polarized ring system [16]. It is also known that in the presence of certain intercalating compounds, as acridine orange and ethidium bromide, the *E. coli* ribosomes degrade more rapidly than in their absence [17].

Finally, in evaluating experiments *in vitro*, it should be considered that concentrations exerting antibacterial and curing effect are more than a hundred times higher than those attained *in vivo* by use of the compounds as neuroleptics [18] and antiderepressants [19]. Elucidation of the mode of action of imipramine, amitriptyline and phenothiazines by use of the bacteria model may open a new way for the planning of new types of chemotherapeutics.

REFERENCES

1. MOLNÁR, J., KIRÁLY, J., MÁNDI, Y.: *Experientia* (Basel) **31**, 444 (1975).
2. MOLNÁR, J., MÁNDI, Y., KIRÁLY, J.: *Acta microbiol. Acad. Sci. hung.* **23**, 45 (1976).
3. MÁNDI, Y., MOLNÁR, J., HOLLAND, I. B., BÉLÁDI, I.: *Genet. Res.* **27**, 109 (1976).
4. FISHMAN, V., GOLDENBERG, H.: *Proc. Soc. exp. Biol. (N.Y.)* **112**, 501 (1963).
5. ALFÖLDI, L., RASKÓ, I., KERÉKES, E.: *J. Bact.* **96**, 1512 (1968).
6. THOMAS, J. O., DEEDS, F., EDDY, J.: *J. Pharmacol. exp. Ther.* **64**, 280 (1938).

7. DEEDS, F., STOCKTON, A. B., THOMAS, J. O.: *J. Pharmacol. exp. Ther.* **65**, 353 (1939).
8. DECOURT, P., GASTAL, R., GRENAT, R.: *C. R. Acad. Sci. (Paris)* **237**, 1109 (1953).
9. BOURDON, J. L.: *Ann. Inst. Pasteur* **101**, 876 (1961).
10. POPPER, M., LORIAN, V.: *Presse méd.* **67**, 212 (1959).
11. GEIGER, H., FINKELSTEIN, D. A.: *Schweiz. med. Wschr.* **84**, 1063 (1954).
12. SPITZ, H., LEVIT, R.: *Schweiz. med. Wschr.* **86**, 869 (1956).
13. HAHN, F. E., CIAK, J.: *Mode of Action of Curing Compounds. Drug-Inactivating Enzymes and Antibiotic Resistance.* Springer, Berlin-Heidelberg-New York, 1975. p. 235.
14. ROTH, J. A., GILLING, C. N.: *Molec. Pharmacol.* **11**, 28 (1975).
15. MÜLLER, W., BÜNEMANN, H., DATTAGUPTA, N.: *Europ. J. Biochem.* **54**, 279 (1975).
16. MÜLLER, W., CROTHERS, D. M.: *Europ. J. Biochem.* **54**, 267 (1975).
17. SURYANARAYANA, T., BURMA, D. P.: *Biochem. biophys. Res. Commun.* **65**, 708 (1975).
18. RIVERA-COLIMLIM, L., CASTANEDA, L., LASAGNA, L.: *Clin. Pharmacol. Ther.* **14**, 978 (1973).
19. ELONEN, E., LINOILA, M., LUKKARI, I., MATTILA, M. J.: *Acta pharm. toxicol.* **37**, 274 (1975).

Address of the authors:

ÓZSEF MOLNÁR, YVETTE MÁNDI
Institute of Microbiology, University Medical School
Dóm tér 10, H-6720 Szeged, Hungary

GYULA SCHNEIDER
Institute of Organic Chemistry, University of Szeged
Dóm tér 8, H-6720 Szeged, Hungary

IAN BARRY HOLLAND
Department of Genetics, School of Biological Sciences, University of Leicester
Adrian Building, University Road
Leicester LE 1 7RH, England

ORAL IMMUNIZATION OF MONKEYS WITH POLYVALENT DYSENTERY VACCINE

E. K. DHIKIDZE, K. N. KAVTARADZE, R. I. KRILOVA, K. RAUSS,
I. KÉTYI and ADELE VERTÉNYI

Institute of Experimental Pathology and Therapy, U.S.S.R. Academy of Medical Sciences, Sukhumi, U.S.S.R. and Institute of Microbiology, University Medical School, Pécs, Hungary

(Received May 5, 1976)

Macaca mulatta monkeys were immunized orally with polyvalent Boivin extract of *Shigella flexneri* 2a, 3a, 4a, 4b, 6 and *Shigella sonnei*. The total immunizing dose for each component was equivalent to 1.2×10^{12} cells. After challenge with 7.5×10^{10} cells of a virulent *S. flexneri* 2a strain, out of 20 immunized animals 2 developed dysentery and 4 showed mild dyspepsia; all 6 control animals became ill with dysentery. Vaccination failed to influence the incidence, duration or the intermittent character of shigella excretion. Protective antibodies appeared in high titre in the serum of immunized animals.

As shown earlier [1-3], oral vaccination with killed *Shigella* cells or with Boivin antigen extract induces protective serum and coproantibodies equivalent in level to those produced after parenteral immunization or natural infection.

In the present study we immunized monkeys and examined the clinical, pathomorphological, bacteriological and immunological changes appearing after challenge with virulent shigellae.

Materials and methods

Experimental animals. *Macaca mulatta* monkeys weighing 2-4 kg were housed singly in separate cages. The monkeys, which had been kept in the Sukhumi Institute for long before starting the experiments, were clinically healthy and bacteriologically free from shigellae and salmonellae.

Vaccine and immunization schedule. Boivin extracts were prepared from *S. flexneri* serotypes 2a, 3a, 4a, 4b and 6 and for *S. sonnei*. The dialysed extracts were precipitated with 4 volumes of ethanol and freeze-dried. The components were standardized by haemagglutination inhibition test and tableted with lactose. In a tablet weighing 0.16-0.18 g, each component was present in about 0.5 mg amount. The tablets were checked with haemagglutination inhibition test after redissolving and their potency was estimated with active mouse protection test [1, 2]. Each Boivin antigen component represented approximately 5.7×10^{10} bacterial cells; the total dose for one animal (10.5 mg) amounted accordingly, to 1.2×10^{12} cells for each component.

The immunization schedule is presented in Table I. Basic immunity was established with four doses given on consecutive days; subsequent doses at weakly intervals served to maintain immunity. The tablets were dissolved in water and given before feeding.

Challenging was performed orally with saline suspension of a virulent *S. flexneri* 2a strain, 13 days after the last immunizing stimulus. The optimum challenging dose (7.5×10^{10} cells) was determined in preliminary experiments.

Bacteriological checking was done several times before and during immunization of every animal. After challenging, the monkeys were, as a rule, examined daily for three weeks,

then 21 subsequent samples were taken at 2-4 day intervals. Rectal swabs were immediately inoculated onto SSC medium (Difco). Colonies suspicious of shigellae were examined by the routine cultural and serological methods.

Clinical and pathomorphological examinations. General condition of the animals was checked and their faeces inspected practically daily for 2 $\frac{1}{2}$ months. Autopsy of every animal dying of dysentery was performed routinely; several monkey dying 5-7 months after challenge were also examined.

Assay of protective antibodies. The passive chick embryo test [4] was used. Dilutions of the serum pools were injected intravenously to 10-day embryos; the challenging dose (*S. flexneri* 2a strain, 20 LD₅₀) was given intravenously about half an hour later. The virulence test was performed at the same time. The results were read after 48 hr.

Statistical analysis. LD₅₀ and ED₅₀ were calculated as described by KÄRBER [5]. Significance was estimated by the χ^2 test.

Table I
Immunization schedule and challenge

Dose, No.	Day	Interval between doses, day	Amount of antigen for each component	
			mg	No. of tablets
1	1	—	0.5	1
2	2	1	1.0	2
3	3	1	1.5	3
4	4	1	1.5	3
5	11	7	1.5	3
6	18	7	1.5	3
7	25	7	1.5	3
8	32	7	1.5	3
	45	13	Challenge with 7.5×10^{10} cells of <i>S. flexneri</i> 2a	

Basic immunity was established with the first 4 doses; weakly doses were given to maintain immunity. The total dose of 10.5 mg antigen for each component represented about 1.2×10^{12} cells.

Results

Twenty animals not harbouring salmonellae and shigellae were immunized as shown in Table I. The immunization did not influence the appetite and activity of the animals and caused no gastro-enteric indisposition. Thirteen days after the last immunizing dose all animals including the six non-immunized controls, were challenged orally with 7.5×10^{10} cells of a virulent *S. flexneri* 2a strain.

Clinical findings after challenge are summarized in Table II. All six control animals developed dysentery. Two of them had a mild form of the disease characterized by loose, watery stools containing mucus and leukocytes (but no blood) and by anorexia and decreased activity lasting for a

Table II

Clinical state during 2½ months observation of immunized and control monkeys after challenge with S. flexneri 2a

Group	No. of animals	Clinical symptoms				
		Symptomless	Intestinal reaction	Mild dysentery	Severe dysentery	Lethal dysentery
Immunized	20	14	4	1	1	1
Control	6	—	—	2	4	1

Intestinal reaction: loose stools for 1 day, general symptoms absent. Mild dysentery: loose stools for 2–4 days, anorexia, inactivity. Severe dysentery: stools with blood, mucus and leukocytes lasting for at least 8 days, severe general condition, sometimes dehydration.

few days. Four animals showed severe dysentery with prolonged excretion of bloody and mucous stools and severe general symptoms; in two of them there was a marked dehydration. In consequence of the latter, one animal died on the 7th day; two monkeys were ill for 8 days, one — with transient remission — showed symptoms of dysentery for 42 days.

Fourteen out of the 20 immunized monkeys remained symptomless, while 4 of them excreted loose stools for 1 day without any general symptoms. These may also be regarded as healthy animals, since the “intestinal disorder” may have been associated with the effect of enterotoxin released from shigellae ingested in great numbers with the challenging dose. Of the two remaining animals one developed mild symptoms, the other a severe dysentery leading to dehydration and death on the 18th day.

The difference between immunized and control animals was striking. If in the immunized group only the 14 symptomless monkeys are regarded as immune to the disease, the difference is significant above the 1% level ($\chi^2 = 9.450$, $p = < 0.01$).

Clinical diagnosis was confirmed by bacteriological findings. After challenge *S. flexneri* 2a was recovered and the animals were free from other enteric pathogens. At necropsy of the two animals, classical morphological signs of dysentery were evident.

After the end of the experiment (2½ months after challenge), although shigella excretion could not be shown, each animal was treated with 0.25 g lincomycin and 0.25 g streptomycin b.i.d. Then the monkeys were used for other kinds of experiments together with non-immunized animals some of which were evidently symptomless carriers of shigellae. Five to 7 months after immunization only 1 out of the 19 immunized monkeys developed dysentery. At necropsy this animal showed signs of dysentery, whereas in 6 sacrificed monkeys of this group dysenteric changes were absent. Two of the 5 animals, which had been used as controls in the vaccination experi-

ments, became ill with clinically and morphologically typical dysentery. These data were confirmed by bacteriological findings.

During the 2½ months observation period after challenge, bacteriological examinations showed the usual intermittent excretion of the pathogenic agent. As expected, there was no difference in development, duration and other characteristics of carriership between the immunized and control groups (Table III).

Table III

Shigella excretion by immunized and control monkeys

Duration of shigella excretion	No. of animals	
	Immunized	Control
Before and during immunization	—	—
After challenge for 1–5 days	8	1
6–10 days	2	4
11–30 days	2	—
31–45 days	8	1
Total	20	6

Blood samples were taken before immunization, on the 4th day and at the end of vaccination and finally on the day of challenge. The sera for each group were pooled at equal amounts and used for passive chick embryo protection test. Challenging was performed with 20 LD₅₀ of a virulent *S. flexneri* 2a strain (LD₅₀ = 3 cells). Results are shown in Table IV.

Table IV

Protective titres in the sera of immunized and control monkeys
Passive chick embryo test; challenging dose, *S. flexneri* 2a 20 LD₅₀

Serum pools	ED ₅₀ (ml)	RE*	χ ²
Non-immunized control animals	>0.05	1	
Immunized animals before vaccination	>0.05	1	χ ² =5.252 P<0.05
Immunized animals, 4th day of vaccination	0.005	>10	
Immunized animals, end of vaccination (32nd day)	0.0005	>100	χ ² =13.594 P<0.001
Immunized animals, day of challenge (45th day)	0.0005	>100	

* relative icieny

Neither the control nor the test animals had detectable amounts of protective antibodies before immunization. Basic immunization resulted in a significant rise of antibodies (χ² = 5.252, p < 0.05) as early as on the 4th

day. At the end of the immunization course and on the day of challenge the protective antibody titre was high ($\chi^2 = 13.594$, $p < 0.001$ as compared to that before immunization).

Discussion

In our latest paper on oral dysentery vaccination [3] we summarized arguments for the use of killed bacteria or antigen extracts as opposed to vaccines containing live attenuated bacteria. FORMAL *et al.* [6] have already shown that avirulent strains with limited multiplication power in the tissues immunize better than killed vaccines only if their penetrating capacity has not been lost. This finding has been confirmed in our laboratory with strains obtained from the Formal team and with our own avirulent mutants [7]. Due to the released endotoxins, such strains, however, cause undesirable side effects in monkeys [8]. As the immune effect of both killed and live vaccines is usually evaluated on the basis of the number of bacteria ingested, it is questionable whether a simple comparison is justified: live vaccines, because of their multiplication in the organism, represent in reality an antigen dose larger than the calculated.

Boivin extracts were absorbed well from the intestines and dysentery vaccines of this type induced excellent immunity with high serum and copro-antibody titres in mice; a similarly good seroconversion was observed in humans of various ages [1-3, 7]. It is unquestionable that the value of a vaccine is confirmed only by positive field trials. Such data are, however, difficult to obtain in countries with low dysentery morbidity. In Hungary, we have attempted vaccination in closed communities where dysentery had been expected to be more prevalent. So far, the sporadic occurrence of the disease even in these communities, has not allowed to collect a sufficient number of data [9].

On the basis of analogous natural susceptibility and clinical, pathological and immunological reactivity, vaccination of monkeys may supply valuable epidemiological data. In practice, however, considering the high increase of susceptibility associated with keeping the animals in captivity, successful experiments are difficult to carry out. According to TAKASAKA *et al.* [10], even under the best conditions, monkeys waiting for air transport in India are infected with shigellae in 13.2% and reaching Japan this ratio increases to 20.1%. Thus, many of the monkeys arriving at experimental institutions have manifest dysentery or are symptomless carriers of shigellae (with the hazard of frequent exacerbation) or may even be resistant to the disease as a result of natural infection.

For our experiments we, therefore, chose healthy and bacteriologically negative animals which had been kept for long in the Sukhumi Institute and were free from protective antibodies. The small number of animals used may

not allow to draw general conclusion. Yet, the clinical resistance of the animals, confirmed by their high protective antibody level, is a good indicator of the efficacy of vaccination. The clinical reactivity of our immunized and control animals agreed well with the experience of FORMAL *et al.* for penetrating live hybrid strains: 2 out of the 24 *Macaca mulatta* monkeys vaccinated with *S. flexneri* 2a, and 19 out of the 24 control animals developed mild or severe dysentery [11], whereas 6 out of 23 monkeys given polyvalent vaccine (*S. flexneri* 1b, 2a, 3 and *S. sonnei*) and 16 out of the 21 corresponding control animals, became ill [12].

The number of virulent bacteria (7.5×10^{10} cells) found earlier and in the present experiments to represent the optimum challenging dose, seemed high, but was almost identical with the dose (5×10^{10}) used by FORMAL *et al.* [11, 12]. In volunteers, DUPONT *et al.* [13] induced dysentery with 10^5 – 10^8 organisms at a maximum of 77%. This ratio could not be increased by higher doses, only the incubation period became shorter and the clinical manifestations more severe. Our challenged monkeys exhibited a short (24 hr) incubation time.

The present results show that oral vaccination with Boivin extracts, which has been used safely in humans of every age group [3] is suitable for producing high seroconversion and efficient clinical resistance to dysentery in monkeys. In view of these findings the vaccine seems to be adequate for immunizing human communities exposed to the disease.

REFERENCES

1. RAUSS, K., PUSZTAI, S., JOÓ, I., KÉTYI, I., MÁTÉ, J.: Acta microbiol. Acad. Sci. hung. **14**, 153 (1967).
2. RAUSS, K., KÉTYI, I., MÁTÉ, J., KNEFFEL, P., AMBÓ, G., MARÓCZI, J., PUSZTAI, S., JOÓ, I., SZENDREI, L.: Acta microbiol. Acad. Sci. hung. **16**, 159 (1969).
3. KÉTYI, I., RAUSS, K., VERTÉNYI, A.: Acta microbiol. Acad. Sci. hung. **21**, 81 (1974).
4. KÉTYI, I.: Zbl. Bakt. I. Abt. Orig. **194**, 332 (1964).
5. KÄRBER, G.: Naunyn-Schmiedeberg's Arch. exp. Path. Pharmacol. **162**, 480 (1931).
6. FORMAL, S. B., LABREC, E. H., KENT, T. H., FALKOW, S.: J. Bact. **89**, 1374 (1965).
7. RAUSS, K., KÉTYI, I., ANGYAL, T.: Acta microbiol. Acad. Sci. hung. **14**, 143 (1967).
8. FORMAL, S. B., LABREC, E. H., PALMER, A., FALKOW, S.: J. Bact. **90**, 63 (1965).
9. RUDNAI, O.: Personal communication.
10. TAKASAKA, M., HONJO, S., FUJIWARA, T., HAGIWARA, T., OGAWA, H., IMAIZUMI, K.: Japan J. Med. Sci. Biol. **17**, 259 (1964).
11. FORMAL, S. B., KENT, T. H., AUSTIN, S., LABREC, E. H.: J. Bact. **91**, 2368 (1966).
12. FORMAL, S. B., KENT, T. H., MAY, H. C., PALMER, A., FALKOW, S., LABREC, E. H.: J. Bact. **92**, 17 (1966).
13. DUPONT, H. L., HORNICK, R. B., DAWKINS, A. T., SNYDER, M. J., FORMAL, S. B.: J. infect. Dis. **119**, 296 (1969).

Address of the authors:

E. K. DHIKIDZE, K. N. KAVTARADZE, R. I. KRILOVA
Institute of Experimental Pathology and Therapy
P.O.B. 66, Gora Trapetziya, Sukhumi, U.S.S.R.

IVÁN KÉTYI, ADELE VERTÉNYI
Institute of Microbiology, University Medical School
Szigeti út 12, H-7643 Pécs, Hungary

EPSTEIN-BARR VIRUS AND CYTOMEGALOVIRUS ANTIBODIES IN INFECTIOUS MONONUCLEOSIS

L. GERGELY, JUDITH CZEGLÉDY, A. SZALKA, L. VÁCZI and L. BINDER

Institute of Microbiology, University Medical School, Debrecen and Hospital for Infectious Diseases "László", Budapest

(Received May 10, 1976)

A method has been evolved for the demonstration of Epstein-Barr virus (EBV) and cytomegalovirus (CMV) infection in 83 cases of infectious mononucleosis. Serum samples were tested for EBV IgM, anti-VCA IgG, anti-EBNA, CMV IgM and CMV IgG antibodies. An acute-phase sample (or samples) and a convalescence sample were examined in each case, and in 44 cases an additional sample was examined 5–12 months after the illness. Since the different antibodies showed characteristic differences in both titre and persistence, a reliable serodiagnosis has become possible. Acute EBV infection is characterized by the presence of EBV-VCA IgG and EBV IgG antibodies and the lack of anti-EBNA. The latter becomes demonstrable as late as in the 4th to 5th month after infection. Mean age of the patients was 19 years. EBV infection was demonstrated in 65%, CMV infection in 18% of the cases. In 12% double infection seemed to be probable.

Epstein-Barr virus (EBV) infection of young adults manifests itself with infectious mononucleosis (IM) in two-thirds of the cases [1]. It depends on the mode of transmission and the immunological status of the infected subject whether or not the syndrome develops [2]. The IM syndrome, especially in cases negative for heterophilic agglutinins, may, however, be caused by other aetiological agent(s) too, such as the cytomegalovirus (CMV) as shown by KLEMOLA *et al.* [3] and confirmed by others. It seemed therefore justified to develop reliable procedures for the demonstration of EBV and/or CMV infection.

The antibodies which appear in all cases of the corresponding infection are

(a) EBV IgM and CMV IgM antibodies, which are the first to appear in the serum during the acute phase as shown by BANATVALA *et al.* [4] for EBV IgM and by KRECH *et al.* [5] for CMV IgM;

(b) anti-VCA (IgG VCA) and CMV IgG antibodies, which persist in the healthy population for life;

(c) anti-EBNA, i.e., the antibody reacting with the EBV nuclear antigen. This antibody appears late during EBV infection and persists for life.

Serial serum samples from patients suffering from IM were tested for these five antibodies. The results are presented below.

Materials and methods

Serum samples. Serum samples were taken from 83 patients suffering from IM. Most of them were residents of Budapest or Pest county. From each of the patients, two or three blood samples were obtained during the acute phase and one to three samples during convalescence. From each of 44 patients, an additional sample was taken 5–12 months after the illness. The sera were kept at -20°C until tested.

Testing for antibodies by immunofluorescence (IF).

1. **EBV-VCA IgG antibody.** Serum samples were prepared from lymphoblastoid cells of the cell line B95-8, a line in which at least 10% of the cells are positive for EBV antigens. (The cell line was kindly supplied by Professor G. KLEIN, Department of Tumour Biology, Karolinska Institutet, Stockholm, Sweden.) The cells were cultivated for 6 days at 37°C in MEM (Minimum Essential Medium) enriched with 10% calf serum. Then the cells were washed twice with BSS (0.8% NaCl, 0.014% CaCl_2 , 0.04% KCl, 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.06% KH_2PO_4 , 0.06% $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, pH 6.9). The suspension contained 10^7 cells/ml. One drop of the suspension was placed on a slide. The smears were fixed in acetone for 10 min, then incubated with the serum to be tested at 37°C in a wet chamber for 30 min. In the second step, the smears were thoroughly washed and stained with FITC-labelled anti-human IgG (Hyland). A 1 : 20 dilution of the reagent was used. After a second incubation as described above, the smears were washed and dried and one drop of glycerol-PBS mixture (1 : 1) was added, whereafter the smears were covered with a coverslip and examined in a Leitz Orthoplan UV microscope equipped with a Ploem Opakilluminator. An objective of $\times 40$ was used. The filter system consisted of 4 mm BG 38 + 1.5 mm BG 12 + an interference plate of AL 470, 495 nm and a final filter K 510. Appropriate positive and negative controls were always included. The highest serum dilution giving specific fluorescence was regarded as serum titre.

2. **CMV IgG antibodies.** Coverslip preparations were made from human embryonic fibroblast cells which had been infected with strain CMV-Ad-169. When about 90% of the cells showed cytopathic changes, the cultures were fixed in acetone for 10 min at room temperature and placed on slides. Subsequently, various dilutions of the serum tested were dropped on the coverslips. The further steps of the procedure were the same as described under [1].

3. **EBV IgM antibodies.** Smears were prepared as described under [2]. We used 1 : 15 and higher serum dilutions. Otherwise, the high IgG concentration would have disturbed the IF reaction. The mixtures were incubated at room temperature for 20 hr and the cells were stained with 1 : 10 diluted FITC-labelled anti-human IgM (Hyland).

4. **CMV IgM antibodies.** Coverslip cultures were prepared as described under [2]. The further procedures were the same as under [3].

5. **Anti-EBNA.** Reedman's anti-complement immunofluorescence method (ACIF) was applied. B95-8 cells were cultured for 5–6 days, then washed twice with BSS. The last sediment was resuspended in 1 ml of ice-cold 0.95% solution of sodium citrate. The suspension was allowed to stand for 1 min, one drop was placed on a precooled slide and immediately dried under a cold air drier. The preparation was fixed in a 1 : 1 mixture of methanol and acetone at -20°C for 10 min. Antibody titration was performed in three steps.

(a) The smears were incubated with different dilutions of the serum tested in a wet chamber at 37°C for 30 min.

(b) The preparations were washed with BSS and then incubated with 1 : 5 diluted fresh human serum negative for EBV antibodies (source of complement) at 37°C for 30 min.

(c) The preparations were washed with BSS, then incubated with a 1 : 20 dilution of FITC-labelled anti-human complement (Hyland) in a wet chamber at 37°C for 30 min. After repeated washings and rinsing with distilled water, one drop of a 1 : 1 mixture of glycerol and PBS was added, the smear was covered with a coverglass and examined as described under [1].

Titration of heterophilic antibodies. The Paul-Bunnell-Davidsohn (PBD) method was applied [10].

Results

The serological results for three typical hospitalized IM cases are presented in Table I.

Table I

Examination of serial serum samples from three patients with IM

Designation of case	PBD titre	Time of sampling*	Anti-EBV titres			Anti-CMV titres	
			anti-VCA IgG	IgM	anti-EBNA	IgG	IgM
1. J. G. ♂	+ (1:1024)	day 0	40	20	5	10	<15
		6	160	80	< 5	10	<15
		12	160	80	< 5	10	<15
		40	80	15	< 5	10	<15
		month 5	40	<15	5	10	<15
		12	40	<15	20	10	<15
2. G. P. ♂	—	day 0	40	<15	20	160	15
		8	40	<15	20	640	40
		38	40	<15	20	320	15
		month 6	40	<15	20	320	<15
		9	40	<15	20	160	<15
3. F. I. ♀	+ (1:64)	day 0	40	15	< 5	80	<15
		4	80	20	< 5	160	15
		8	80	20	< 5	320	40
		30	160	15	< 5	160	15
		month 4	40	<15	5	160	<15
		9	40	<15	20	80	<15

* Day 0 is the day of admission or the subsequent day.

Case 1. PBD: positive. Anti-EBV-VCA: a 4-fold rise from 1 : 40 to 1 : 160 by the 12th day after admission (the day of discharge) and a subsequent fall to 1 : 40 by the 40th day; the titre remained at this level for at least a year. EBV IgM antibody: a 4-fold increase in the first week was followed by a fall to the threshold of detectability by the 40th day and disappearance by the end of the 5th month: the CMV antibody status did not change during the one-year observation period.

Case 2. PBD: negative. The CMV IgG antibody titre was considerable in the first sample, yet, became still higher within eight days, then tended downward to reach the initial level nine months after admission. The CMV IgM titre ran parallel with the CMV IgG at a much lower level and these antibodies disappeared between the 38th day and the 6th month. EBV IgM antibodies did not appear during the observation period and the other EBV antibodies did not change in titre.

Case 3. PBD: positive. Both the anti-EBV-VCA IgG and the anti-CMV IgG antibodies showed an initial increase and a subsequent decline. The two IgM antibodies displayed a similar tendency at a much lower level. Anti-EBNA was first demonstrated four months after admission, to increase in titre during the subsequent five months.

There was no doubt that in Case 1 the EBV and in Case 2 the CMV was the aetiological agent. In Case 3 a double infection was demonstrated.

In Tables II-V the frequencies of the combined presence or absence of different antibodies are presented.

The majority of the serum samples taken on admission were positive for anti-EBV-VCA IgG and negative for anti-EBNA, viz., 80% of the PBD-positive sera and 70% of the PBD-positive ones (Table II). All the sera containing no anti-EBV VCA IgG were negative for anti-EBNA (Table III).

Table II

Combined presence of EBV-VCA IgG and EBNA antibodies in acute phase sera

EBV-VCA IgG	Anti-EBNA	PBD positive	PBD negative
positive ($\geq 1:10$)	negative ($< 1:5$)	45	22
negative ($< 1:10$)	positive ($\geq 1:5$)	0	0
positive ($\geq 1:10$)	positive ($\geq 1:5$)	6	6
negative ($< 1:10$)	negative ($< 1:5$)	1	3
Total		52	31

Table III

Combined presence of EBV-VCA IgG and EBV IgM antibody in acute-phase sera

EBV-VCA IgG	EBV IgM	PBD positive	PBD negative
4-fold rise in titre or a $> 1:160$ titre	positive ($\geq 1:15$)	33	7
1:160 anti-EBNA positive	negative ($< 1:15$)	2	2
1:10-1:40 no anti-EBNA	positive ($\geq 1:15$)	8	2
Total		43	11

In the majority of the cases in which a 4-fold rise in the anti-EBV-VCA IgG titre was demonstrated, the EBV IgM antibody was also present (first of all in the PBD-positive sera). We found only four acute-phase serum samples in which, beside a high anti-EBV-VCA IgG titre, there was no EBV

IgM but anti-EBNA could be detected. A few samples contained low-titre anti-EBV-VCA IgG besides EBV IgM antibodies (Table IV).

Table IV

Combined presence of anti-EBNA and EBV IgM antibody in acute-phase sera

Anti-EBNA		EBV IgM	PBD positive	PBD negative
positive	($\geq 1:5$)	negative ($< 1:15$)	15	18
negative	($< 1:5$)	positive ($\geq 1:15$)	37	10
positive	($\geq 1:5$)	positive ($\geq 1:15$)	0	0
negative	($< 1:5$)	negative ($< 1:15$)	0	3
Total			52	31

Anti-EBNA and EBV IgM antibodies could never be detected in the same sample, whereas acute-phase sera negative for anti-EBNA and positive for EBV IgM antibody occurred in 75% of the PBD-positive and 32% of the PBD-negative sera (Table V).

Table V

Incidence of anti-EBV IgM in acute-phase sera positive for anti-EBV-VCA IgG and negative for anti-EBNA

No. of anti-VCA positive anti-EBNA negative sera	EBV IgM positive		EBV IgM negative	
	PBD positive	PBD negative	PBD positive	PBD negative
67	43	21	1	2

In 95% of the anti-EBV-VCA IgG positive and anti-EBNA negative acute-phase sera, virus specific IgM was also present. On the other hand, testing of 44 patients several months after their illness has shown that those who had been positive for anti-EBV-VCA IgG and anti-EBV IgM and negative for anti-EBNA in the acute phase remained anti-EBV-VCA IgG positive, but became positive for anti-EBNA and negative for EBV IgM.

Discussion

We tested serial serum samples of 83 IM patients with the aim to demonstrate the EBV or CMV origin of the cases. During the acute phase 77% of the cases were positive for anti-EBV-VCA IgG and EBV IgM and negative for anti-EBNA. This serological pattern, in itself is indicative of a primary

EBV infection. From half of these cases an additional sample was available several months after onset. In these cases a seroreversion for EBV IgM and a seroconversion for anti-EBNA brought further evidence of the EBV aetiology.

The results of serial testings were in accordance with those reported by other authors, but they followed their patients only for one or another of the antibodies investigated in the present study. HENLE *et al.* [11], NIEDERMAN *et al.* [12] and EVANS *et al.* [13] have shown that IM due to EBV cannot develop in subjects having anti-VCA antibodies and the patients negative for this antibody in the acute stage show seroconversion later with a gradual increase of the VCA antibody titre. Other authors [4, 14, 15] demonstrated EBV IgM antibodies in the acute phase of IM. According to HENLE *et al.* [6] the anti-EBNA requires several weeks, or even months, to develop.

Several authors [5, 16] have found CMV IgM antibodies in the early phase of CMV infection. In our patients the appearance and the dynamics of CMV IgG antibodies were in accordance with literary data.

The three cases described above are good examples of fresh EBV infection (Case 1), a fresh CMV infection (Case 2) and a double infection (Case 3). The 83 patients under study have been grouped by the serodiagnostic result. The data in Table VI support the suggestion that the PBD-positive IM is of EBV origin, whereas a PBD-negative case may equally be caused by EBV or CMV. In 10 cases a double infection seems to have occurred.

Table VI
Serodiagnosis of 83 IM patients

	Fresh EBV infection	Fresh CMV infection	Supposedly double infection	Infection not proved	Total
PBD positive	40	—	9	3	52
PBD negative	14	15	1	1	31

The follow-up of the dynamics of the antibody titres after the acute phase (clinical recovery) did not alter the diagnosis that had been established on the basis of the early titres; it only served for checking the diagnosis. In practice, there is no reason to examine convalescent sera, as the patients, except in the infrequent complicated cases, stay only two weeks in hospital. In this two-week period it is nevertheless advisable to examine several consecutive blood samples.

Our data suggest that simultaneous titration of several antibodies in more than one sample is necessary for determining whether the existing antibodies originate from a fresh infection or from a previous one.

In the four cases shown in Table II, where besides anti-EBV VCA IgG, no EBV IgM could be detected in the acute-phase sample, the presence of anti-EBNA indicated that the EBV infection had occurred earlier.

In another 10 cases, in which the anti-EBV-VCA IgG titre was low, the presence of EBV IgM and the absence of anti-EBNA pointed to a fresh EBV infection.

It may be concluded that determination of the anti-EBV-VCA IgG antibody alone does not provide a firm basis for the aetiological diagnosis of IM. A high titre of this antibody may be present without fresh infection and its low titres do not exclude a fresh EBV infection.

Acknowledgement. The authors are indebted to Mr. J. MÁRTON for excellent technical assistance.

REFERENCES

1. NIEDERMAN, J. C., EVANS, A. S., SUBRAHMANYAN, C., McCOLLUM, R. W.: *New Engl. J. Med.* **282**, 361 (1970).
2. EPSTEIN, M. A., ACHONG, B. G.: *Lancet* **2**, 836 (1973).
3. KLEMOLA, E., KÄÄRIÄINEN, L., VON ESSEN, R., HALTIA, K., KOIVANIEMI, A.: *Acta med. scand.* **182**, 311 (1967).
4. BANATVALA, J. E., BEST, J. M., WALLER, D. K.: *Lancet* **1**, 1205 (1972).
5. KRECH, U. H., JUNG, M., JUNG, F.: *Cytomegalovirus infection in men*. Karger, Basel 1971. p. 26.
6. HENLE, G., HENLE, W., HORWITZ, CH. A.: *J. infect. Dis.* **130**, 231 (1974).
7. HENLE, G., HENLE, W.: *J. Bact.* **91**, 1248 (1966).
8. MILLER, G., SHOPE, T., LISCO, H., STITT, D., LIPMAN, M.: *Proc. nat. Acad. Sci. (Wash.)* **69**, 383 (1972).
9. REEDMAN, B. M., KLEIN, G.: *Int. J. Cancer* **11**, 499 (1973).
10. DAVIDSOHN, I., WALKER, P. H.: *Amer. J. clin. Path.* **5**, 455 (1935).
11. HENLE, G., HENLE, W., DIEHL, Y.: *Proc. nat. Acad. Sci. (Wash.)* **59**, 94 (1968).
12. NIEDERMAN, J. C., McCOLLUM, R. W., HENLE, G., HENLE, W.: *J. Amer. Med. Ass.* **203**, 205 (1968).
13. EVANS, A. S., NIEDERMAN, J. C., McCOLLUM, R. W.: *New Engl. J. Med.* **279**, 1121 (1968).
14. SCHMITZ, H., SCHERER, M.: *Arch. ges. Virusforsch.* **37**, 332 (1972).
15. HAMPAR, B., HSU, K. C., MARTOS, L. M., WALTER, J. L.: *Proc. nat. Acad. Sci. (Wash.)* **68**, 1407 (1971).
16. SCHMITZ, H., HAAS, R.: *Arch. ges. Virusforsch.* **37**, 131 (1972).

Address of the authors:

LAJOS GERGELY, JUDITH CZEGLÉDY, LAJOS VÁCZI
Institute of Microbiology, University Medical School
4012 Debrecen, P.O.B. 17, Hungary

LÁSZLÓ BINDER, ANDRÁS SZALKA
Hospital for Infectious Diseases, "László"
1450 Budapest, P.O.B. 29, Hungary

CYTOMEGALOVIRUS-INDUCED MEMBRANE ANTIGENS IN PRODUCTIVELY INFECTED CELLS

I. BOLDOGH, ÉVA GÖNCZÖL and L. VÁCZI

Institute of Microbiology, University Medical School, Debrecen

(Received May 18, 1976)

Adsorbed but not penetrated virus can be removed from the CMV-infected cell membrane by digestion with cystine-activated papain. Membrane antigens appear on 80–90% of the infected cells 14–20 hr after infection as a result of *de novo* protein synthesis. Antigen synthesis can be blocked with inhibitors of protein synthesis, but not with DNA inhibitors. In the early stage of infection, pooled human convalescent serum reacted well with the membrane antigen, whereas pooled antiserum of rabbits immunized with CMV virion suspension gave a positive reaction with a small proportion of the cells. After the 48th hr, both the human and the rabbit serum pool reacted with the membrane of the infected cells. Absorption with cells cultured for 24 hr after CMV infection reduced the neutralization titres of the antisera only slightly but the titre reduction was considerable when absorption was performed with cells cultured for more than 48 hr after infection. It is concluded that on the membrane of cells productively infected by CMV at least two membrane antigens are present, one coded for by the DNA of the parent virus and another which is the product of the DNA of the virus progeny. The two antigens can be differentiated serologically.

Virus-specific membrane antigens (MA) have been detected on the surface of cells infected by DNA viruses. Some of these viruses are [1–3], others are not oncogenic [4, 5]. The presence of viral antigens on the surface of tumour cells is of special importance because the specific immune response may favourably influence the prognosis of the tumour disease [6, 7]. On the surface of CMV-transformed cells, there appear virus-specific antigens [8], which are also present on the productively transformed cells [9]. It has been reported [10] that in certain clones of CMV-transformed cells only viral MAs can be demonstrated in a high percentage of the cells, intracellular CMV antigens occur rarely.

Since some relationship has been found between CMV and certain human tumours [11–13], an analysis of the CMV-induced surface antigens seemed to be of interest.

In the experiments to be reported we have shown that cells productively infected by CMV are able to synthesize two qualitatively different MAs, an early and a late antigen.

Materials and methods

Cell cultures. Secondary human embryonic fibroblast cultures (HEF) were used throughout. The cultures were grown and maintained in Minimum Essential Medium (MEM) supplemented with 10% bovine serum, 0.125% NaHCO₃, 100 I.U./ml penicillin and 100 µg/ml strepto-

mycin. To prevent mycoplasma contamination, 100 $\mu\text{g/ml}$ of kanamycin was added to the medium at intervals.

Virus. The AD-169 strain of human cytomegalovirus (CMV) was kindly supplied by Dr. H. K. ANDERSEN (Institute of Microbiology, University of Medicine, Aarhus, Denmark) in 1973. Since then it has been maintained by continuous propagation in HEF cells. The titre of the virus was determined as described by PLUMMER and BENYESH-MELNICK [14].

Antisera. CMV-specific hyperimmune rabbit serum was produced as described by ANDERSEN [15, 16], with the only modification that the virus suspension to be inoculated was inactivated by UV irradiation (Hanau H. K80 distance, 28 cm; 30 min). The antiserum used in the experiments was a pool of four rabbit sera. It had an anti-MA titre of 1 : 360 and a neutralization titre of 1 : 280. We also used a human anti-CMV serum. This was a pool of sera from six CMV-positive subjects and had an anti-MA titre of 1 : 480 and a neutralization titre of 1 : 360. The antisera were absorbed with 5×10^8 uninfected HEF cells/ml before use.

Indirect immunofluorescence (IF). To detect MA and intracellular antigens, we used infected live and acetone-fixed (fixation, 3 min at room temperature) HEF cultures. The methods have been published earlier [17, 18].

Enzymatic removal of antigens. The method published by TURNER *et al.* [19] was adopted. Some details of the procedure are described with the experiments.

Neutralization test. The plaque-reduction test [14, 16] was used.

Absorption of antisera. Cell cultures were digested with papain and washed five times with PBS 24, 40, 96 or 144 hr after infection with CMV. The cells were scraped off from the glass wall with a rubber rod, suspended in PBS and centrifuged at 4 °C for 10 min at 1000 rpm. The intact cells thus obtained were suspended in antiserum (5×10^8 /ml), and then kept at 37 °C for 1 hr and, subsequently at 2 °C for 20 hr while shaken up at intervals. The absorbed serum was centrifuged at 2000 rpm for 15 min and stored at -20 °C until use.

Specification of reagents and drugs. Ara-C (Sigma Chemical Co., St. Louis, Mo., U.S.A.); Cycloheximide (Calbiochem, Los Angeles, Calif., U.S.A.); Papain (Serva, Entwicklungslabor, Heidelberg, Germany); Cystine (Reanal, Budapest, Hungary); MEM (Lyophilized, Gibco Grand Island Biological Company, Oakland, Calif., U.S.A.); Conjugates, anti-human IgG and anti-rabbit IgG (Hyland Division Travelon Laboratories, Costa Mesa, Calif., U.S.A.); Agar Noble special (Difco Laboratories, Detroit, Mich., U.S.A.).

Results

Enzymatic removal of adsorbed virus from the surface of infected cells.

Suspended HEF cells, 10^7 /ml, with CMV added were kept in a CO₂ incubator at 37 °C for 1 hr. The multiplicity of infection was 1–2 p.f.u./cell. To remove the non-adsorbed virus, the cells were washed twice with PBS and incubated in an enzyme solution at 37 °C. The following enzyme solutions were used in parallel experiments: cystine-activated papain in PBS (0.1, 0.3 and 0.4%), trypsin (0.025%), a 4 : 1 mixture of trypsin (0.025%) and versene (0.005%). Further samples of the cell suspension were washed twice or five times with PBS instead of enzyme treatment. The papain and trypsin effect was suspended by adding 2 mM iodoacetate and 2% calf serum (final concentrations), respectively. After washing twice with PBS and centrifugation at 1000 rpm for 5 min, the cells were examined for IF. Results are shown in Fig. 1.

Ninety to 95% of the adsorbed virus was removed from the cell surface during a 60 min incubation in 0.3 or 0.4% cystine-activated papain (Fig. 1/a). The capacity of the cells to attach to the glass surface was only slightly reduced by this treatment.

A sample of untreated infected cells was washed five times with PBS. Almost all of these cells remained MA-positive, whereas a 2 $\times$ 15 min treat-

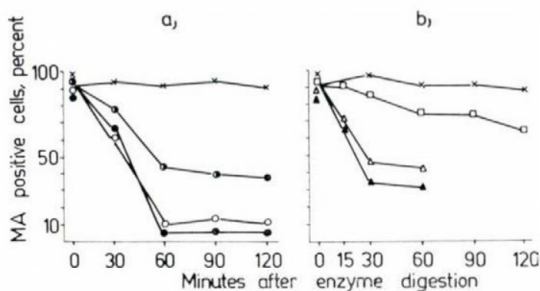


Fig. 1. Removal by enzyme treatment of the membrane antigenicity due to adsorbed virus. Cystine activated papain: ●—● 0.1%; ○—○ 0.3%; ●—● 0.4%; △—△ 0.025% trypsin; ▲—▲ 4:1 mixture of 0.025% trypsin solution and 0.005% versene solution; ×—× washing with PBS twice at 30 min interval; □—□ washing with PBS five times at 30 min intervals

ment with trypsin or trypsin + versene rendered 50–60% of the cells free of MA (Fig. 1/b). A prolonged trypsin or trypsin + versene treatment reduced the viability of the cells considerably.

In further experiments, the cells were digested for 60 min in 0.3% cystine-activated papain. This technique allowed to examine the MA synthesized *de novo*.

Detectability of early and late MA(s) with human and rabbit antiserum. In coverslip cultures the attachment of cells was reduced by 5–10% and their replication delayed by 8–12 hr by pretreatment with 0.3% papain. This difference did not considerably influence the further experiments, as only the attached cells were tested for newly-appearing MA. For this purpose pooled antisera of human and rabbit origin were used. The use of pools was necessary because of the variability of the individual immune response, *viz.*, some anti-MA components may be lacking in individual CMV-positive human sera.

To detect MAs and intracellular viral antigens we used coverslip cultures either without fixation or after fixation in acetone for 3 min. Results are shown in Fig. 2.

As to the MAs synthesized *de novo*, there was a striking difference between the results obtained by the use of human antiserum (Fig. 2/a) and those obtained with rabbit antiserum (Fig. 2/b). A similar difference was not shown by the virus antigen adsorbed to the cell surface.

With human antiserum we were able to demonstrate surface antigens appearing 12–14 hr after infection. These reached the highest prevalence (on 80–90% of the cells) at 20–24 hr. This percentage was not influenced by 15 µg/ml ara-C in the medium. In the presence of the protein inhibitor cycloheximide (10 µg/ml), however, these antigens did not appear.

When undigested cells were cultured in the presence of ara-C, the percentage fluorescence decreased till the 10th–12th hr, then began to rise. In the

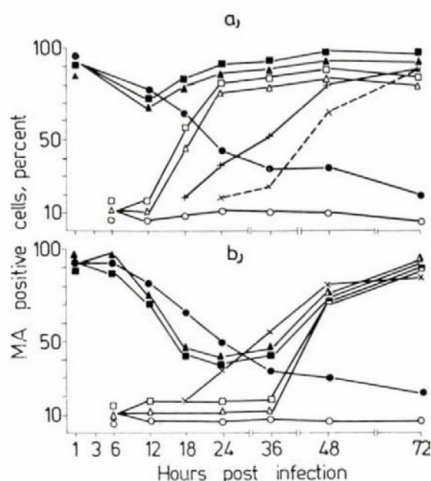


Fig. 2. MA-positive cells in per cent at different times post infection. As test serum, pooled anti-CMV positive human serum (a) and pooled serum of artificially infected rabbits (b) was used. Cells without enzyme treatment: $\blacktriangle$ — $\blacktriangle$ 15 μ g/ml ara-C; $\bullet$ — $\bullet$ 10 μ g/ml cycloheximide; $\blacksquare$ — $\blacksquare$ no drug. Papain-digested cells: $\triangle$ — $\triangle$ 15 μ g/ml ara-C; $\circ$ — $\circ$ 10 μ g/ml cycloheximide; $\square$ — $\square$ no drug. $\times$ — $\times$ Cells showing cytopathic effect; $\times$ — $\times$ cells showing early nuclear antigen

presence of cycloheximide the membrane fluorescence fell to 20% by the 72nd hr, indicating that a great part of the adsorbed virus had disappeared.

When rabbit antiserum was used with the papain-digested cells, only 8–15% of the cells showed fluorescence in the first 36 hr. This percentage was influenced neither by ara-C nor by cycloheximide. After the 36th hr, the percentage positivity began to rise, and this rise could be prevented with cycloheximide, but not with ara-C.

The membrane fluorescence of the undigested cells stained with rabbit antiserum declined till the 24th hr, then increased until the 48th hr. Thereafter the course of the curves in Fig. 2/b was similar to that in Fig. 2/a.

Our results have raised the idea that the antigens appearing on the surface of the cells are qualitatively inhomogeneous. We performed serum absorption experiments to check this suggestion.

Absorption of neutralizing and anti-MA IF antibodies by MAs. Samples from the pooled human and rabbit antisera were absorbed with CMV-infected cells incubated for different periods of time after infection. 5×10^8 infected cells/ml serum were added and the neutralization and anti-MA titres of the absorbed sera were determined. Results are shown in Table I.

Absorption with cells incubated for 24 hr after infection did not reduce the titres to a considerable degree. If the cells had been incubated for 48 hr before use, the titres were significantly reduced by the absorption, but a second absorption left the residual titres nearly unchanged. The presence or

Table I
Absorption of CMV antisera with CMV membrane antigens

Absorbing HEF cells	NT*	anti-MA**	NT	anti-MA
	titre of human serum		titre of rabbit serum	
noninfected	360	480	280	360
24 hr p.i.***	320	480	280	360
24 hr p.i.	320	440	240	320
48 hr p.i.	120	160	120	160
48 hr p.i.	100	120	100	120
48 hr p.i.+ara-C	120	160	120	160
96 hr p.i.	40	40	20	20
144 hr p.i.	20	20	20	20

* NT = anti-CMV neutralization titre

** anti-MA = indirect IF reaction tested with 144 hr cells

*** p.i. = after infection with CMV

absence of ara-C during postinfection cultivation did not influence the results, indicating that the viral DNA was synthesized after the 48th hr. Absorption with cells incubated for 96 or 144 hr reduced the titres to 1/9–1/24 of the original.

It seems therefore reasonable to differentiate between an early MA (EMA), i.e., a MA appearing in the first 48 hr of postinfection incubation and a late MA (LMA) which appears after the 48th hr. The synthesis of EMA does not, while that of LMA does, require *de novo* DNA synthesis.

Discussion

SCHWARTZ and NATHENSON [21] and TURNER *et al.* [19] were the first to remove HLA antigens from the cell membrane by digestion with papain. We have shown that the membrane antigenicity which appears soon after CMV adsorption can be removed by incubation in a 0.3% solution of cystine-activated papain, without causing any remarkable cell damage. Part of this very early membrane antigenicity could be removed by trypsin digestion or incubation in a trypsin–versene mixture as well, but treatment for more than 30 min damaged the cells considerably.

After the surface antigenicity due to the adsorbed virus had been removed, an investigation of the *de novo* synthesis of MAs became possible.

The surface antigenicity due to the adsorbed virus can be detected with human and rabbit antisera. However, the *de novo* synthesized MAs appearing on the papain-treated cells between the 12th and 36th hr after

infection need human antiserum for being detected. The MAs appearing later react with both antisera. The different behaviour of the two antisera might be explained by the different circumstances of immunization, *viz.*, the human antibodies were produced against the replicating virus, whereas the antibodies in the rabbit immune serum had been induced mainly by virion components.

PEARSON *et al.* [22] have shown that the neutralizing and anti-MA titres of EBV immune sera closely correlate with each other, suggesting that the MAs contain peplon antigens, among others. Similar observations have been reported by ROIZMAN and SPRING [23] for herpes simplex virus (HSV) and by ANDERSEN [24] for CMV. These data are supported by our results presented in Table I. The neutralization titres and the IF titres to the early MAs of the antisera were reduced by more than 50% by absorption with antigens already present on the cultured cells 48 hr after infection, and the residual titres were only slightly reduced by a second absorption with 48 hr cells. Nevertheless, these absorbed sera gave positive membrane fluorescence in 90–100% of the virion-producing cells being in a more advanced phase of the replication cycle. This indicates that certain antibodies had not been removed from the sera by the absorption.

Absorption of sera with cells carrying late MAs (96–144 hr cells) decreased both the neutralization and anti-late MA titres 1/15–1/20 of the original.

Thus, appearance of the whole MA spectrum of the infected cells seems to need a contribution by the DNA of the virus progeny.

The above results support our view that the MA(s) induced by the parent virus are different from those coded for by the virus progeny. The qualitative difference between EMA and LMA was proved by the following observations.

1. The residual neutralization and anti-MA titres of antisera once absorbed with EMA were not reduced by a second absorption with 48 hr cells, whether or not these had been incubated with ara-C.

2. The MAs on 90–100% of the 120–144 hr cells gave positive IF reaction with antisera fully adsorbed with 48 hr cells.

3. The neutralizing and anti-MA antibodies needed absorption with virion-producing 120–144 hr cells for being removed completely.

These results are in accordance with data previously reported by other authors. MA was demonstrated on HSV-infected cells early in the reproduction cycle, before infectious virus had been synthesized. Production of this MA could be inhibited with protein inhibitors, but not with ara-C [25]. In the EBV-carrying Raji cells MA is formed before DNA synthesis sets in [26]. On EBV-infected cells, ERNBERG *et al.* [27] detected virus-specific EMA and a serologically distinguishable LMA. Of these, EMA was demonstrable only on virus-non-producing (VCA-negative) cells, whereas the LMA only on VCA-positive cells. SILVESTRE *et al.* [28], using ferritin-labelled EBV antibodies, detected LMA only on cells positive for EBV antigens (VCA and EA), whereas

EMA antigenicity was observed only on the cells free from these antigens. Both MAs were, however, found in the peplon of the extracellular virion. According to the cross-blocking experiments of SVEDMYR *et al.* [29], the EBV MA consists of at least two, more probably three, serologically distinguishable components.

It deserves special attention that the appearance of EMA is independent of DNA synthesis. On the surface of CMV-transformed cells, virus-specific MA(s) are synthesized, and the same antigens are present among the MAs of the productively infected cells [9]. The surface antigens of these tumour cells may be regarded as early products of the virus genome, since all efforts have failed to demonstrate viral DNA synthesis in the CMV-transformed cells [8]. Early MA has been detected in cells either infected or transformed by polyoma virus or SV₄₀ [30-32].

The membrane fluorescence that appeared in our experiments very early, 12 to 24 hr after infection, deserves special interest because it was detectable with our pooled human antiserum, but not with the pooled rabbit antiserum, and in the serum absorption test the early membrane fluorescence did not react with the majority of the neutralizing and anti-MA antibodies. Taking into account that these antigens do not react with our pooled rabbit serum we suppose that they are lacking or are present only in traces in the CMV virion and they are not synthesized in rabbits artificially immunized with virion suspensions. As an alternative possibility, the specific immune response may be determined by the host species.

Our results seem to be consistent with the possible appearance of non-virion membrane antigens after CMV infection. Although we have not found a similar suggestion for the herpesvirus family in the literature, the existence of such antigen(s) cannot be excluded without further investigations.

The relationship of CMV with infectious mononucleosis [12] and certain solid tumours [13] has been suggested recently. Furthermore, CMV shares some properties with viruses that proved to be oncogenic, e.g., stimulation of the host DNA and RNA synthesis [33-35], raising of the mitotic index [36] and transformation of cells *in vitro* [8]. Thus, an analysis of the viral antigens appearing on the surface of CMV-infected cells should not be neglected. These antigens may be of both theoretical and practical importance.

REFERENCES

1. ZUR HAUSEN, H., SCHULTE-HOLTHAUSEN, H., KLEIN, G., HENLE, G., HENLE, W., CLIFFORD, P., SANTESSON, L.: *Nature (Lond.)* **228**, 1056 (1970).
2. LUNGER, P. D.: *Virology* **24**, 138 (1970).
3. BLACK, P. H.: *Ann. Rev. Microbiol.* **22**, 391 (1968).
4. UEDA, Y., ITO, M., TAGAYA, I.: *Virology* **33**, 180 (1969).
5. MIYAMOTO, H., KATO, S.: *Biken J.* **11**, 343 (1968).
6. TEVETHIA, S. S., DIAMONDPOULOS, G. T., RAPP, F., ENDERS, J. F.: *J. Immun.* **119**, 1192 (1968).

7. DUFF, R., DOLLER, E., RAPP, F.: *Science* **180**, 79 (1973).
8. ALBRECHT, T., RAPP, F.: *Virology* **55**, 53 (1973).
9. LAUSCH, R. N., MURASKO, M. D., ALBRECHT, T., RAPP, F.: *J. Immunol.* **112**, 1680 (1974).
10. NACHTIGAL, M., ALBRECHT, T., RAPP, F.: *Intervirology* **4**, 77 (1974).
11. DUVALL, C. P., CASAZZA, A. R., GRIMLEY, P. M., CARBON, P. P., ROWE, W. P.: *Ann. intern. Med.* **64**, 531 (1966).
12. KLEMOLA, E., VON ESSEN, R., WAGER, O., HALTIA, K., KOIVUNIEMI, A., SALMI, I.: *Ann. intern. Med.* **71**, 11 (1969).
13. GIRALDO, G., BETH, E., HAGUENAU, F.: *J. natl. Cancer Inst.* **49**, 1509 (1972).
14. PLUMMER, G., BENYESH-MELNICK, M.: *Proc. Soc. exp. Biol. (N.Y.)* **117**, 145 (1964).
15. ANDERSEN, H. K.: *Arch. ges. Virusforsch.* **36**, 133 (1972).
16. ANDERSEN, H. K.: *Arch. ges. Virusforsch.* **35**, 143 (1971).
17. GÉDER, L., VÁCZI, L., JENEY, E., GÖNCZÖL, É., LEHEL, F.: *Acta microbiol. Acad. Sci. hung.* **13**, 365 (1966/67).
18. GÖNCZÖL, É., ANDERSEN, H. K.: *Arch. ges. Virusforsch.* **44**, 147 (1974).
19. TURNER, M. J., STROMINGER, I. L., SANDERSON, A. R.: *Proc. nat. Acad. Sci. (Wash.)* **69**, 200 (1972).
20. SVEDMYR, A., DEMISSIE, A., KLEIN, G.: *J. nat. Cancer Inst.* **44**, 595 (1970).
21. SCHWARTZ, B., NATHENSON, S. G.: *Transplant. Proc.* **3**, 180 (1970).
22. PEARSON, G., DEWEY, F., KLEIN, G., HENLE, G., HENLE, W.: *J. nat. Cancer Inst.* **45**, 989 (1970).
23. ROIZMAN, B., SPRING, S. B.: *Proc. Conference on Cross Reading Antigens and Neoantigen Williams and Wilkins, Baltimore, Md. 1967*, p. 85.
24. ANDERSEN, H. K.: *Arch. ges. Virusforsch.* **38**, 297 (1972).
25. ITO, M., BARRON, A. L.: *J. Immunol.* **108**, 711 (1972).
26. GERGELY, L., KLEIN, G., ERNBERG, I.: *Virology* **45**, 10 (1971).
27. ERNBERG, I., KLEIN, G., KOURILSKY, F. M., SILVESTRE, D.: *J. nat. Cancer Inst.* **53**, 61 (1974).
28. SILVESTRE, D., ERNBERG, I., NEAUPORT-SAUTES, C., KOURILSKY, F. M., KLEIN, G.: *J. nat. Cancer Inst.* **53**, 67 (1974).
29. SVEDMYR, A., DEMISSIE, A., KLEIN, G., CLIFFORD, P.: *J. nat. Cancer Inst.* **44**, 610 (1970).
30. DEICHMANN, G. I.: *Adv. Cancer Res.* **12**, 101 (1969).
31. MEYER, G., LHERISSON-STABONI, A. M., BONNEAU, H.: *Int. J. Cancer* **4**, 520 (1969).
32. RAPP, F., BUTEL, J. S., FELDMAN, L. A., KITAHARA, T., MELNICK, J. L.: *J. exp. Med.* **121**, 935 (1965).
33. JEOR, S. C. ST., ALBRECHT, B. T., FUNK, F. D., RAPP, F.: *J. Virology* **13**, 353 (1974).
34. ALBRECHT, T., NACHTIGAL, M., JEOR, S. C. ST., RAPP, F.: *J. gen. Virol.* **30**, 167 (1976).
35. TANAKA, S., FURUKAWA, T., PLOTKIN, A. S.: *J. Virology* **15**, 297 (1975).

Address of the authors:

ISTVÁN BOLDOGH, ÉVA GÖNCZÖL, LAJOS VÁCZI
 Institute of Microbiology, University Medical School
 H-4012 Debrecen, P.O.B. 17, Hungary

MACROPHAGE DISAPPEARANCE REACTION IN *RANA ESCULENTA* INDUCED BY SPECIFIC ANTIGEN AND RABBIT LYMPHOKINES

S. SIPKA, I. BOLDOGH and T. SZILÁGYI

*Institute of Pathophysiology and Institute of Microbiology, University Medical School,
Debrecen*

(Received May 25, 1976)

Macrophage disappearance reaction was induced by intraperitoneal injections of specific antigens in *Rana esculenta* sensitized by bovine gamma globulin and *Mycobacterium bovis* BCG. Rabbit lymphokines derived from concanavalin A stimulated blood lymphocytes injected intraperitoneally were able to induce macrophage disappearance reaction in normal *Rana esculenta*. This suggests that mammalian lymphokines are capable of acting in amphibia. Mammalian lymphokines have not an exclusive class-specificity in vertebrates.

There are few data about the delayed type hypersensitivity in amphibia [1–3].

Macrophage disappearance reaction (MDR) is accepted as a model of delayed hypersensitivity in mammals [4]. NELSON and NORTH [4] presented evidence that the loss of macrophages in MDR is due to their adherence to the lining of the peritoneal cavity. SONOZAKI and COHEN [5] found that MDR is mediated by a soluble antigen-activated lymphocyte factor, by a lymphokine or lymphokines. Lymphokines are biologically active products of thymus-derived (T) lymphocytes produced by specific antigen or nonspecific mitogenic agents e.g. by phytohemagglutinin or concanavalin A [6]. YOSHIDA *et al.* [7] demonstrated that lymphokines have no species specificity.

In this work we examined MDR induced by specific antigen in sensitized *Rana esculenta*, then we imitated MDR in normal *Rana esculenta* by intraperitoneal injection of lymphokines produced by concanavalin A stimulated rabbit lymphocytes.

Material and methods

Animals. Adult frogs (*Rana esculenta*) weighing 50–80 g of both sexes held in wet conditions at 16–19 °C were used in the experiments for sensitization in December and January, for testing MDR in February and March.

Adult New Zealand rabbits of both sexes weighing 2.5–3 kg were used for preparation of lymphocytes.

Antigens and immunization. Ten mg of bovine gamma globulin (BGG) prepared in our laboratory were injected in 0.25 ml saline together with an equal volume of complete Freund's adjuvant into the dorsal lymph sac of frogs.

Lyophilized BCG vaccine (5×10^5 bacteria, 0.5 ml; National Institute of Hygiene, Budapest) was injected together with an equal volume of complete Freund's adjuvant into the dorsal lymph sac of frogs.

Preparation of lymphokines. Lymphocytes were separated from heparinized rabbit blood in Uromiro Ficoll solution by centrifugation at 800 g for 15 min at room temperature.

Lymphocytes (10^7) were cultured at 37 °C and in 5% CO_2 atmosphere, together with 20 μg of concanavalin A (Sigma) for 24 hr in Parker's medium completed with streptomycin (100 μg per ml) and penicillin (100 IU/ml). No serum was added. Cell free concanavalin A-containing supernatants of concanavalin A-stimulated rabbit lymphocytes were used as lymphokines. The samples had macrophage migration inhibition factor activity as tested on guinea pig macrophages, and skin reactive factor activity as tested on rat skin.

Macrophage disappearance reaction. Frogs sensitized by BGG (group II) and BCG (group III) after 4–5 weeks were tested for MDR by intraperitoneal injection of 6 μg BGG and 1 IU of tuberculin (National Institute of Hygiene, Budapest). To induce macrophage-rich peritoneal exudate, animals were treated intraperitoneally with 2 ml of paraffin oil 5–7 days before the MDR reaction.

Four hr after the antigen injection the animals were bled and their peritoneal cavity was washed with 4 ml of frog Parker (80 ml Parker + 20 ml distilled water) containing 3% fetal calf serum anticoagulated by heparin (1 IU/ml). All procedures took place in plastic tubes and dishes. Peritoneal exudates containing macrophages were centrifuged and washed three times with frog Parker solution. Macrophages were collected in standard volumes and evaluated quantitatively and qualitatively. The number of cells was estimated in Buerker chambers and differential cell counts were made after May–Grünwald–Giemsa staining.

Rabbit lymphokines in a volume of 0.2 ml were injected intraperitoneally to the frogs (group V). MDR was tested after 4 hr.

In control experiments (group I), the animals were treated intraperitoneally with Parker solution corresponding in volume to the antigen injected in groups II and III. In the experiments with lymphokines the control animals were injected intraperitoneally with the supernatants of cultured lymphocytes completed with concanavalin A after cultivation, and containing the same quantity of concanavalin A as the lymphokine samples (group IV). Every experimental group contained at least 10 animals.

Results

Both specific antigens (BGG, BCG) were able to produce MDR in the sensitized animals and rabbit lymphokines in normal frogs. They induced a remarkable decrease in the number of macrophages in the peritoneal fluid of frogs (Table I).

Table I

Macrophage disappearance reaction induced by specific antigens and by rabbit lymphokines in Rana esculenta

Groups of animals	Macrophage disappearance reaction	
	macrophages/ml (mean $\pm$ SD)	lymphocytes/ml (mean $\pm$ SD)
I Control	$1.70 \pm 0.15 \times 10^6$	$0.64 \pm 0.12 \times 10^6$
II BGG sensitized	$0.87 \pm 0.10 \times 10^6$	$1.09 \pm 0.12 \times 10^6$
III BCG sensitized	$0.21 \pm 0.10 \times 10^6$	$1.05 \pm 0.10 \times 10^6$
IV Lymphokine control	$2.00 \pm 0.16 \times 10^6$	$0.68 \pm 0.11 \times 10^6$
V Lymphokine treated	$1.25 \pm 0.14 \times 10^6$	$0.70 \pm 0.10 \times 10^6$

All values are averages of at least three experiments.

Discussion

In our experiments MDR seemed to be a sensitive model for testing delayed type hypersensitivity in sensitized *Rana esculenta*. Mitogen-induced rabbit lymphokines imitated MDR in normal *Rana esculenta*. In all experi-

mental groups there was a marked decrease in the number of macrophages. In two experimental groups (II, III) the number of lymphocytes increased somewhat in MDR. This effect was not detectable in the group treated with lymphokines. No explanation can be offered for the effect.

It was remarkable that the MDR mediating effect of mammalian lymphokines should manifested itself in amphibia. Beside the lack of species specificity, mammalian lymphokines do not seem to have an exclusive class-specificity in vertebrates.

REFERENCES

1. HILDEMAN, W. H., HAAS, R.: J. Immunol. **83**, 478 (1959).
2. COHEN, N.: J. exp. Zool. **187**, 37 (1968).
3. DRÖSSLER, K., AMBROSIUS, H.: Acta biol. med. germ. **29**, 441 (1972).
4. NELSON, D. S., NORTH, R.: Lab. Invest. **14**, 89 (1965).
5. SONOZAKI, H., COHEN, S.: Cell. Immunol. **2**, 341 (1971).
6. PICK, E., TURK, J. L.: Clin. exp. Immunol. **10**, 1 (1972).
7. YOSHIDA, T., NAGAI, R., HASHIMOTO, T.: Lab. Invest. **29**, 329 (1973).

Address of the authors:

SÁNDOR SÍPKA, TIBOR SZILÁGYI

Institute of Pathophysiology, University Medical School
H-4012 Debrecen, P.O.B. 23, Hungary

ISTVÁN BOLDOGH

Institute of Microbiology, University Medical School
H-4012 Debrecen, P.O.B. 17, Hungary

CROSS-NEUTRALIZATION REACTIONS OF ESCHERICHIA COLI AND VIBRIO CHOLERAЕ ENTEROTOXINS AS STUDIED BY RABBIT SKIN INOCULATION TEST

AJAIB SINGH, K. S. MOHAN, S. K. SETHI and D. V. VADEHRA

Department of Microbiology, Panjab University, Chandigarh, India

(Received July 12, 1976)

Neutralization of enterotoxins of *Vibrio cholerae* 569 B and *Escherichia coli* 1046. by antitoxins to *V. cholerae* 569 B, *E. coli* 334, 408-3 and 10407 was studied by intradermal inoculation test in the rabbit. Neutralization of *V. cholerae* enterotoxin by homologous as well as heterologous antisera of *E. coli* was observed, except that there was no neutralization of the enterotoxin by antiserum to *E. coli* 408-3 enterotoxin. Neutralization of *E. coli* enterotoxin to a varied extent by homologous as well as all heterologous antisera, including that of *V. cholerae* 569 B antitoxin, was also observed.

Enterotoxins of several *Escherichia coli* strains which cause an acute accumulation of fluid in the ileal loop of animal models, like that of *Vibrio cholerae* were shown to be implicated in adult cases of diarrhoea [1–3]. The few reports on cross-neutralization reactions of enterotoxins of *E. coli* in the rabbit ileum seem to be contradictory [2, 4, 5]. Similarly, studies on the immunologic resemblance of *E. coli* and *V. cholerae* enterotoxins [2, 6] could not clearly be interpreted because of the diversity of the antigen preparations used and the low titres of antibodies to enterotoxin tested in the rabbit ileum [5].

We have examined the antigenic relationships of *E. coli* and *V. cholerae* enterotoxins by employing uniform methods of preparation of antigens both in *E. coli* and *V. cholerae*, and attempted to find relationships by cross-neutralization studies in rabbit skin instead of rabbit ileum in view of the greater sensitivity and better resolution of enterotoxic activity in the former system [7, 8].

Materials and methods

Bacterial cultures. Enteropathogenic *E. coli* strains 334 (O15:H12), 408-3 (O18:H12) and 10407 (O78:H11) were obtained from Dr. BRADLEY Sack, Baltimore City Hospital, Baltimore, U.S.A. The strains were isolated from adult humans suffering from cholera-like diarrhoea.

V. cholerae strain 569 B, Inaba was obtained from the Central Research Institute, Kasauli, India. All cultures were maintained on nutrient agar slants and stored at 4 °C.

Preparation of enterotoxins. Enterotoxins of *E. coli* were prepared by the method of EVANS *et al.* [9] described in detail elsewhere [10]. Composition of the culture medium was altered by increasing the concentration of yeast extract from 0.15% to 0.6%, as a high percentage of yeast extract was shown to increase the permeability factor in the enterotoxin [11].

Enterotoxin of *V. cholerae* was prepared by the method of FINKELSTEIN and LOSPALUTO [12] modified as described earlier [10].

Protein estimation. Total protein in the enterotoxin preparations was estimated by the method of LOWRY et al. [13] by using crystalline bovine serum albumin as standard.

Assay of enterotoxin activity in rabbit skin. In order to determine the challenge dose of enterotoxin preparations required for neutralization, serially diluted enterotoxins 0.1 ml in volume were injected intradermally into the back of rabbits. The response was determined as suggested by EVANS et al. [11]. Volumes of 0.1 ml each of 0.05 M phosphate buffer and 0.02 M hydroxymethyl aminomethane-chloride buffer injected intradermally served as controls for *V. cholerae* and *E. coli* enterotoxins, respectively.

The diameter of the reaction was plotted against the dilution of the injected enterotoxin and the challenge dose was selected as the concentration of enterotoxin (protein) corresponding to the distal point in the linear portion of the curve.

Antitoxin preparation. The concentration of protein in the *E. coli* and *V. cholerae* enterotoxin preparations was adjusted to 5 mg and 750 µg per ml, respectively and used as antigen for raising antitoxic serum in rabbits.

Injection schedule. The first two doses consisted of 0.3 ml of antigen and an equal volume of complete Freund's adjuvant injected intraperitoneally one week apart. Then subcutaneous injections of 0.8 ml of antigen were given weekly for five weeks. A booster injection containing 0.8 ml of toxin was given intravenously after another 15 days and 7 days later the animals were bled. Pooled serum from 2-3 animals from each group was preserved with merthiolate (1:10 000) at 4 °C after determining the antibody titre of pooled antisera from each group by BOYDEN's passive haemagglutination test [14].

Toxin-antitoxin titration in rabbit skin. The slightly modified method of EVANS et al. [15] for determining serum antitoxic activity from blueing plus induration diameter of the reaction was employed for toxin-antitoxin titration. Each enterotoxin preparation was diluted so as to contain one challenge dose per 0.05 ml volume, then 0.2 ml of the toxin was mixed with equal volumes each of serially diluted (three-fold dilutions ranging from 0 to 6561) antiserum inactivated at 56 °C for 30 min. The tubes were incubated in a water bath at 37 °C for 30 min. After incubation 0.1 ml each of the mixed content was injected intradermally and the reaction was recorded. Controls consisted of intradermal injections of one challenge dose of toxin in 0.1 ml volume and 0.1 ml of undiluted antiserum.

Titration of antitoxins involving neutralization of the skin reaction was performed by using homologous as well as heterologous antisera as described above.

Results

Passive haemagglutination test was primarily used to detect the presence of antibodies to various enterotoxins employed to raise the antiserum. The antiserum titres of *E. coli* 334, 408-3, 10407 and *V. cholerae* 569 B were 1280, 640, 640 and 2560, respectively.

One intracutaneous blueing dose was expressed [16] as the amount of choleragen capable of eliciting a reaction 8 mm in diameter. For *E. coli* 10407 enterotoxin, we have measured the diameter of induration extending beyond the faint blueing central zone at the site of inoculation, as this was shown to correlate well with the enterotoxic activity of *E. coli* enterotoxins [11].

Figs 1 and 2 show the dose-response curves of the skin reactions to *V. cholerae* 569 B and *E. coli* 10407 enterotoxin in 10-fold dilution for the former and 2-fold dilution for the latter preparation. The challenging doses used for neutralization studies consisted of 7.5 and 62.5 µg of protein concentration of enterotoxins of *V. cholerae* and *E. coli*, respectively. As determined

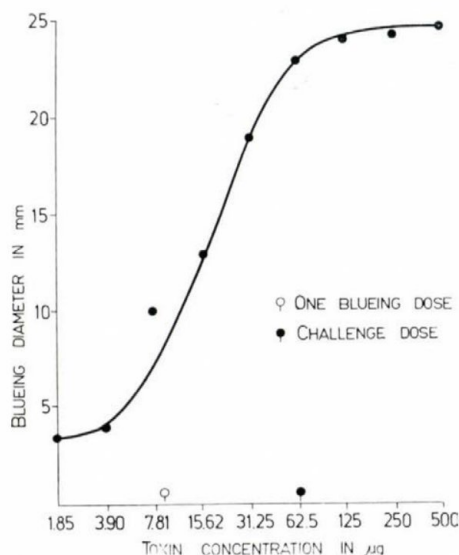


Fig. 1. Dose response curve for *V. cholerae* 569 B enterotoxin

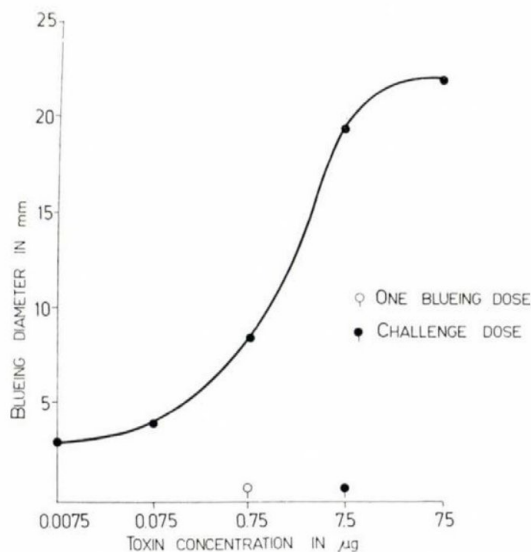


Fig. 2. Dose response curve for *E. coli* 10407 enterotoxin

from Figs 1 and 2, these corresponded to 10 blueing doses of the former and 7 blueing doses of the latter enterotoxin.

Figs 3 and 4 show the neutralization skin reaction to *V. cholerae* (10 blueing doses) and *E. coli* (7 blueing doses) enterotoxins, respectively by 3-fold dilutions of antisera.

Dilutions of different antitoxins showing 100% as well as 50% neutralization of *V. cholerae* 569 B enterotoxin is seen in Table I. The antitoxic titre at 50% neutralization was calculated by taking into consideration that 0.05 ml of antitoxin was able to neutralize 10 blueing doses (in 0.05 ml) of entero-

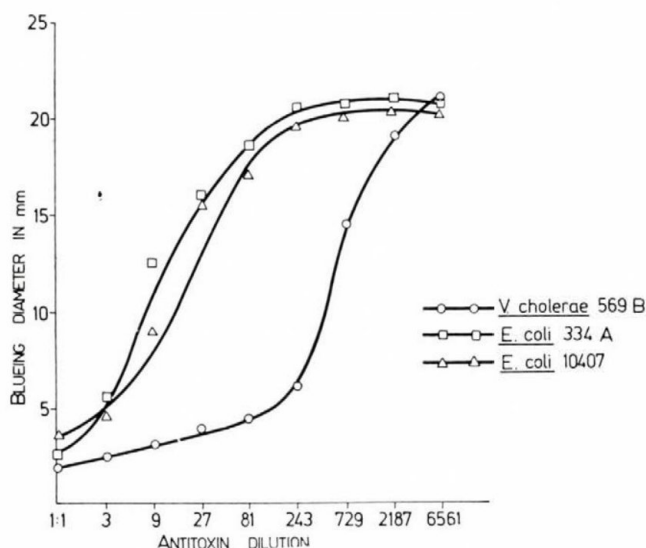


Fig. 3. Neutralization of *V. cholerae* 569 B enterotoxin by antisera to *E. coli* 334, 10407 and *V. cholerae* 569 B enterotoxins in rabbit skin

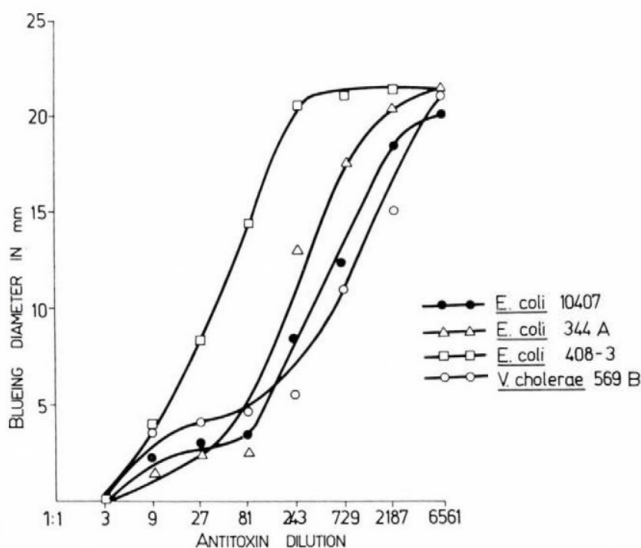


Fig. 4. Neutralization of *E. coli* 10407 enterotoxin by antisera to *E. coli* 10407, 334, 408-3 and *V. cholerae* 569 B enterotoxins in rabbit skin

toxin at a particular dilution. That dilution was multiplied by 200 ($2 \times 10 \times 10$) to determine the antitoxin units per ml. Similarly, Table II exhibits 100% and 50% neutralization of 7 blueing doses of *E. coli* 10407 enterotoxin by different antisera. The neutralization titre was calculated as above except that the dilution of the enterotoxin at 50% neutralization was multiplied by 140 ($2 \times 7 \times 10$) because 7 blueing doses constituted the challenge dose in this case.

Table I

Neutralization of cutaneous response to V. cholerae enterotoxin (10 blueing doses) by various antisera

Antitoxic serum	Antitoxin dilution at complete neutralization	Antitoxin dilution at 50% neutralization	Antitoxic titre at 50% neutralization, units/ml
<i>V. cholerae</i> 569 B	243	729	146×10^3
<i>E. coli</i> 334	3	27	5.4×10^3
<i>E. coli</i> 10407	3	9	1.8×10^3
<i>E. coli</i> 408-3	Nil	Nil	Nil

Table II

Neutralization of cutaneous response to E. coli 10407 enterotoxin (7 blueing doses) by various antisera

Antitoxic serum	Antitoxin dilution at complete neutralization	Antitoxin dilution at 50% neutralization	Antitoxic titre at 50% neutralization, units/ml
<i>V. cholerae</i> 569 B	243	729	102×10^3
<i>E. coli</i> 334	81	243	34×10^3
<i>E. coli</i> 10407	81	729	102×10^3
<i>E. coli</i> 408-3	9	81	11.3×10^3

Discussion

The permeability activity was shown to depend on the diarrhoeagenic activity of the enterotoxin of *E. coli* [11, 15], and can therefore be employed for the detection of antitoxic activity. With the method employed in the preparation of *E. coli* enterotoxins in the present study, both heat stable (ST) and heat-labile (LT) forms could be expected to be present. As the ST fraction does not contribute to the permeability factor activity [11], the reactions observed could be attributed to the LT fraction of *E. coli* enterotoxin.

A high antitoxin titre of *V. cholerae* 569 B at 50% neutralization could be expected of a homologous toxin-antitoxin reaction. It was interesting to

note that *E. coli* 334 and 10407 antitoxins could also neutralize *V. cholerae* 569 B enterotoxin in the rabbit skin although to a lesser extent. Our findings are not in agreement with the observations made by GYLES [17] who reported that cholera toxin was not neutralized by antisera to *E. coli* enterotoxin when titrated in rabbit ileal loops. The small extent of cross-neutralization observed in our study could be attributed to the greater sensitivity of the skin reaction to enterotoxin which has been described as 500–1000 times more sensitive [7] than the rabbit ileal loop model. Attempts to demonstrate cross-neutralization between *V. cholerae* 569 B enterotoxin and antitoxin to *E. coli* 408-3 have, however, failed.

Cross-neutralization of *E. coli* 10407 enterotoxin and antitoxins to *E. coli* 334, 408-3 in the rabbit skin could be indicative of common antigenic determinants of the enterotoxins studied in spite of the diverse serotypes of *E. coli* used for enterotoxin production. The immunological similarity of enterotoxins from serologically different *E. coli* can be expected since several workers [18–20] have reported that the genetic factor controlling production of enterotoxin can be transferred by conjugation between various *E. coli* strains. Cross-neutralization of *E. coli* enterotoxin by *V. cholerae* antitoxin has also been reported [15, 17, 21].

Our investigation has clearly demonstrated that determination of antiserum dilution at 50% neutralization provided a better expression of the neutralization reaction than that at 100% neutralization as suggested by EVANS *et al.* [15]. It is obvious from Figs 3 and 4 that neutralization reaction is not an all or none phenomenon but occurs gradually with the increase of antitoxin concentration. Moreover, a blueing response less than 8 mm in diameter cannot be regarded as significant because it cannot be fitted well into the linear portion of the curve. Thus, neutralization occurring at any point below this level of blueing response is not a true index of the neutralization level.

Thus, neutralization of the cutaneous response to either *V. cholerae* or *E. coli* enterotoxin by homologous antiserum can be employed for estimation of the antitoxin titre at 50% neutralization, to determine the level of antitoxic immune response. The test can also be used effectively to determine the extent of cross-neutralization of enterotoxin by different antitoxins, since it is possible to detect the reaction even when it manifests in a slighter degree.

REFERENCES

1. GORBACH, S. L., BANWELL, J. G., JACOB, B., CHATTERJEE, B. D., MITRA, R. C., BRIGHAM, K. L., NEOGY, K. N.: *J. infect. Dis.* **121**, 38 (1970).
2. SACK, R. B., GORBACH, S. L., BANWELL, J. C., JACOB, B., CHATTERJEE, B. D., MITRA, R. C.: *I. infect. Dis.* **123**, 378 (1971).
3. NALIN, D. R., RICHARDSON, S. M.: *Lancet* **1**, 677 (1973).
4. ETKIN, S., GORBACH, S. L.: *J. Lab. clin. Med.* **78**, 81 (1971).

5. SMITH, H. W., SACK, R. B.: J. infect. Dis. **127**, 164 (1973).
6. GYLES, C. L., BARNUM, D. A.: J. infect. Dis. **120**, 419 (1969).
7. DUHAMAL, R. C., TALBOT, P., GRADY, G. F.: J. infect. Dis. **121**, (Suppl.) S-85 (1970).
8. SINGH, A., GAIND, S., SETHI, S. K.: Zbl. Bakt. I. Abt. Orig. **359**, 288 (1976).
9. EVANS, D. G., EVANS, Jr., D. J., PIERCE, N. F.: Infect. Immun. **7**, 873 (1973).
10. SINGH, A., GAIND, S., SETHI, S. K.: Ind. J. med. Res. **64**, 410 (1976).
11. EVANS, Jr., D. J., EVANS, D. G., GORBACH, S. L.: Infect. Immun. **8**, 725 (1973).
12. FINKELSTEIN, R. A., LoSPALLUTO, J. J. L.: J. exp. Med. **130**, 185 (1969).
13. LOWRY, O. H., ROSENBERG, N. J., FARR, A. L., RANDALL, R. J.: J. biol. Chem. **193**, 265 (1951).
14. BOYDEN, S. V.: J. exp. Med. **93**, 107 (1951).
15. EVANS, D. G., EVANS, Jr., D. J., GORBACH, S. L.: Infect. Immun. **8**, 731 (1973).
16. CRAIG, J. P.: Nature (Lond.) **207**, 614 (1965).
17. GYLES, C. L.: J. infect. Dis. **129**, 277 (1974).
18. SMITH, H. W., HALL, S.: J. gen. Microbiol. **52**, 319 (1968).
19. SMITH, H. W., LINGWOOD, M. A.: J. med. Microbiol. **4**, 301 (1971).
20. SKERMAN, F. J., FORMAL, S. B., FALKOW, S.: Infect. Immun. **5**, 622 (1972).
21. SMITH, H. W., SACK, R. B.: J. infect. Dis. **127**, 164 (1973).

Address of the authors:

AJAIB SINGH, K. S. MOHAN, S. K. SETHI, D. V. VADEHRA
Department of Microbiology, Panjab University
Chandigarh-160 014, India

**SEVENTH CONGRESS OF THE
HUNGARIAN SOCIETY OF MICROBIOLOGY**

BUDAPEST, SEPTEMBER 6-9, 1976

ABSTRACTS OF PAPERS

Plenary Session

TWENTY-FIVE YEARS OF THE HUNGARIAN SOCIETY OF MICROBIOLOGY

E. FARKAS

National Institute of Hygiene, Budapest

In the late forties of this century, the microbiologist members of the re-organized Hungarian Academy of Sciences, R. MANNINGER, G. IVÁNOVICS and A. HAVAS (D. FEHÉR joined them somewhat later) and the parasitologist S. KOTLÁN felt the necessity of a society of experts working in different fields of microbiology. Thus, the Hungarian Society of Microbiology (H.S.M.) was founded under the auspices of the Hungarian Academy of Sciences 25 years ago. According to the first constitution of the H.S.M., its main task was to advance general microbiology, virology, bacteriology, protozoology, helminthology, acarо-entomology, immunology and epidemiology. For this purpose, experts interested in practical and theoretical problems of the application of these sciences in the field of human medicine, veterinary medicine, soil research, nutrition research and industry are united in a society in order to deepen their knowledge and promote an interchange of their experiences and experimental results. Helminthology and acarо-entomology was ruled out of the list in 1964, when the Society of Hungarian Parasitologists had been founded.

It has been a great advantage for the H.S.M. that the first president, A. HAVAS, was at the same time director-general of the National Institute of Hygiene (O.K.I.), the institution that has played a central role in the organization of the medical application of microbiology in this country. O.K.I. has intensively supported the activities of the H.S.M. The general secretaries have been elected out of the O.K.I. microbiologists.

In the scientific annual meetings of the H.S.M. all branches of microbiology were represented. Seven large congresses have been organized with international participation. In each of these, 20 or more foreign participants were guests of the H.S.M. By these invitations we made attempts to return the great hospitality enjoyed by our members when they participated at congresses abroad, most frequently in the neighbouring countries.

The H.S.M. joined the International Association of Microbiological Societies in 1966. The representatives in different committees have been G. IVÁNOVICS, K. RAUSS, HEDDA MILCH, I. NÁSZ, E. K. NOVÁK and B. LÁNYI,

among others. The Society is member of the European Society against Viral Diseases (former name, European Society against Poliomyelitis). I. DÖMÖK has been appointed member of the presidium of this Society. From 1958 until the elimination of poliomyelitis in Europe, H.S.M. had a Poliomyelitis Committee which took part, in co-operation with the mentioned European Society, in the organization of the highly successful fight against poliomyelitis in Hungary. I. JOÓ has been elected a member of the Council of the International Association of Biological Standardization.

The local organization of the 2nd International Congress for Virology (1971), of the 13th International Congress of Biological Standardization (1973) as well as of the Symposium on Pyrogenicity etc. of Biological Preparations to be held in this week has been undertaken by the H.S.M., the last two by our Section of Immunology.

We intend to strengthen our international activities and make them more systematic. The considerable number of participants in this Congress from abroad, among them representatives of several microbiological societies, is promising in this respect.

Recently the annual meetings of the H.S.M. have been held in country towns such as Debrecen, Szeged, Kőszeg, Veszprém, Pécs and Sopron. These have been our most successful meetings.

H.S.M. organized several symposia and conferences in which recommendations have been issued in some topics of microbiological nature, having nation-wide importance.

Presidents of the H.S.M. were A. HAVAS (1951–1954), G. IVÁNOVICS (1954–1958 and 1967–1975), R. MANNINGER (1958–1967) and L. VÁCZI (1975–). Secretary-generals: E. FARKAS (1951–1959 and 1966–1975), GY. WEISZFEILER (1959–1966) and F. FÖRNÖSI (1975–). Vice-presidents of the Society: J. MÉSZÁROS and E. FARKAS (1975–).

In the course of years, the H.S.M. formed several sections. At present there are 5 sections, viz. the Section of Mycology (chairmen, J. BÁNHEGYI, 1956–1975 and E. K. NOVÁK, 1975–), Section of Immunology (L. KESZTYÜS, 1958–1972, I. JOÓ, 1972–), Section of Bacteriology (I. KÉTYI, 1975–), Section of Virology (I. DÖMÖK, 1975–), and the Section of Agricultural and Industrial Microbiology (M. KECSKÉS, 1975–).

Among the sections, the most active was the Section of Immunology. Its activity extended to all fields of immunology (initially even to allergology) till 1971. Then the Hungarian Society of Immunology was founded, and since then the activities of the Section of Immunology have only covered immunity against infectious diseases, problems of vaccinations and standardization of immunological procedures.

The H.S.M. worked under the supervision of the Hungarian Academy of Sciences till 1966. Then we joined the Federation of Hungarian Medical

Societies, which is under the supervision of the Ministry of Health, as somewhat more than half of the members belong to the medical profession. Of course, microbiologists of other professions, veterinaries and biologists, are always represented in our presidium and committees.

Members of the H.S.M. exerted an intense activity in the Editorial Board of the *Acta Microbiologica Academiae Scientiarum Hungaricae*. The editors-in-chief were A. HAVAS (1954), G. IVÁNOVICS (1955–1976) and B. LÁNYI (1976–).

H.S.M. has supported microbiologists in attending scientific meetings in this country and abroad. We have offered prizes for papers of our young members and founded the R. MANNINGER medal. This was awarded to 8 microbiologists before the present Congress.

ULTRASTRUCTURE OF THE ACTINOMYCETES OF THE GENUS STREPTOMYCES IN THE COURSE OF ANTIBIOTIC ACCUMULATION AND TRANSPORT

W. KURYLOWICZ

National Institute of Hygiene, Warsaw, Poland

Morphologic observations and ultrastructural assays of microorganisms have seemingly been dominated by the advances in molecular biology. The microbial anatomy and function of cellular organelles have been learned in detail. Information is, however, rather scarce as to the ultrastructural changes accompanying cell function, and their interplay. Referring to the results on biosynthesis of antibiotics, their major part being reckoned among the secondary metabolites, an attempt was made to obtain more integrated information on the correlation of events in antibiotic biosynthesis and the possible ultrastructural cell modifications. As models for such observations, some *Streptomyces* species were selected, producing about 70% of the antibiotics known to date, as well as *Penicillium chrysogenum* producing benzyl penicillin. Each of the genera (strains) was tested under identical standardized conditions. Two mutants of each strain were assayed, those producing large, and those producing small amounts of the antibiotic. Observations and tests were performed with the material from submerged cultures in laboratory and industrial fermentations. Specimens were taken every 24 hr during the antibiotic biosynthesis cycle. The mycelium surface was assayed by scanning microscopy and the microbial surface and ultrastructure by electron microscopy. In the period of elevated biosynthesis of the secondary metabolites, the presence of amorphous or crystal-like structures was found on the mycelium surface, in which 30 to 70% of the antibiotic produced was detected.

In the same period, observation of cell ultrastructure showed in *Streptomyces* numerous membranaceous structures and in *Penicillium chrysogenum* numerous Golgi cisterns. The latter were found by enzymatic and immunological methods to contain the produced benzyl penicillin. The site of antibiotic biosynthesis in the cell, and its transport from cell to the medium, could be determined in *Streptomyces melanochromogenes* and *Penicillium chrysogenum* as models.

SURVEILLANCE OF R-PLASMIDS

H. RISCHÉ, H. TSCHÄPE and W. WITTE

Institute of Experimental Epidemiology, Wernigerode, G.D.R.

Surveillance of the occurrence and distribution of R-plasmids in bacterial populations in the biotic and abiotic environment is a precondition for chemotherapeutic drug policy. The surveillance of R-plasmids consists of (1.) ecological and epidemiological investigations. (1.1.) Prevalence of R-plasmids in pathogenic and apathogenic bacteria occurring in the biotic environment (man, animal); in the abiotic environment (sewage, food, feed); under high selection pressure (hospital, animal production, plant production). (2.) Biological investigations. (2.1.) Genetic properties of R-plasmids. (2.2.) Plasmid-induced variations of the properties of the host bacteria (virulence, phage pattern, antigenic pattern, serological properties). Genetic and ecological investigations allow the conclusion that the distribution of R-plasmids in Gram-negative bacteria differs both in quality and in quantity from the distribution in Gram-positive cocci. The problems of plasmid mediated drug-resistance can only be solved by a chemotherapeutic drug policy with due regard to the different ecological processes. The antibiotic policy has the following purposes. (1.) Estimation and prognosis of the use of chemotherapeutics in the therapy of man and animals, the prophylaxis of animals, animal production and paratherapeutic fields of application (feed industry, cosmetics). (2.) Methods for the limitation of the spreading of infectious drug resistance. (3.) Production and import of chemotherapeutic drugs, antibiotics included. (4.) Development of new drugs for application in medicine, veterinary medicine and other fields. The drug policy is based upon the surveillance of infectious drug resistance and drug monitoring (effectiveness, economy and side effects of the drugs).

BACTERIAL ENDOTOXIN: PRESENT ASPECTS OF ITS CHEMISTRY
AND BIOLOGY

(Lecture given to honour the late Professor K. Rauss)

O. WESTPHAL

Max Planck Institute of Immunobiology, Freiburg, G.F.R.

Endotoxin has been extracted from many S forms of Gram-negative organisms, especially *Enterobacteriaceae* (*Salmonella*, *Escherichia coli*, etc.) and characterized as lipopolysaccharide (LPS) in which the polysaccharide carries the specific determinants of the O-antigen (Kauffmann-White scheme of *Salmonella*, etc.) while the lipid component — lipid A — carries the structures responsible for endotoxic activity (O. LÜDERITZ *et al.*). Pure lipid A was prepared from rough strains such as *Salmonella* R_e mutants, and analysed chemically (E. TH. RIETSCHEL *et al.*). It has a backbone of phosphorylated (β -1,6-)disaccharide which is highly substituted by long-chain fatty acids and hydroxy acids. Being anionic in character due to the phosphoric acid ester groups, it was found that biological activities of the neutral salt forms of lipid A and LPS are dependent of the cation present (CH. GALANOS *et al.*). Salts with organic cations, like triethyl ammonium, are water-soluble, highly dispersed and biologically most active. As an example, the minimum intravenous pyrogenic dose (MPD) in rabbits is in the order of 0.1 nanogram/kg.

With the advanced elaboration of the structure and physicochemistry of lipid A it is possible to manufacture standard endotoxic preparations, and some of the actions of endotoxin can now be reinvestigated under these aspects, such as its pyrogenicity, B lymphocyte mitogenicity, or its impressive tumour-necrotizing effect.

A lipid A vaccine has been produced (CH. GALANOS *et al.*) by which animals can be immunized against certain endotoxic determinants. Lipid A antibodies exert various anti-endotoxic properties under controlled conditions; they also play an important role in endotoxin tolerance (E. TH. RIETSCHEL *et al.*).

Investigations into the mechanism of action and the fate of ever-present endotoxin in higher animals and man, its biochemical and biological dynamics, are now made with comparative studies in highly endotoxin sensitive (rabbit, horse, man) and insensitive species (baboon, vervet, certain strains of mice).

The comparative chemical and biological analysis of lipid A from bacteria remote from *Enterobacteriaceae* will allow to form an idea about the evolution of the endotoxic principle in the vast field of Gram-negative bacteria.

Immunology

PERTUSSIS VACCINE — FACT AND FICTION

J. CAMERON and D. W. STAINER

Connaught Laboratories Limited, Willowdale, Ontario, Canada

The paper concerns both the growth and metabolism of *Bordetella pertussis* and the manufacture and control of pertussis (whooping cough) vaccine. Developments in growth media are reviewed briefly from isolation on Bordet-Gengou medium in 1906 to growth in chemically defined media in the 1960s. Some of the points on the metabolism of the organism which still continue to puzzle investigators are also discussed, for example, conditions of growth in liquid and on solid media, the role played by starch and blood, the effect of fatty acids on growth, and essential growth requirements. In the case of vaccine manufacture, the problems of strain selection, colonial variation and fermenter compared with solid-surface growth are discussed. On the control side, the mouse-protection test is reviewed together with the possible role of agglutinin production as a guide to potency and the role of adjuvants. The value of the mouse-toxicity test is also discussed and its relative performance when applied to cholera and typhoid vaccines. Two factors which affect the performance of the test are reviewed, the mouse strain and the environmental conditions.

ACTIVE IMMUNIZATION AGAINST WHOOPING COUGH

N. KÖHLER-KUBELKA and T. MANHALTER

Institute of Immunology, Zagreb, Yugoslavia

In order to estimate the protective value of pertussis vaccine in the territory of Croatia, morbidity data were analysed over a period of 20 years (1955–1975). The comparison of whooping cough morbidity in a five-year period before the introduction of compulsory immunization and in two five-year periods after the introduction of immunization shows a highly significant decrease. Parallel with the decrease of morbidity, in the Zagreb district there was a decrease in the number of microbiological examinations for whooping cough as well as in the number of positive findings. A total of 844 microbiologically positive cases of pertussis was analysed with regard to vaccination in the period 1960 to 1975.

COMBINED EFFECT OF PERTUSSIS VACCINE AND DIANHYDRODULCITOL
IN MICE

P. ANDERLIK and I. SZERI

Institute of Microbiology, Semmelweis University Medical School, Budapest

Dianhydrodulcitol (DAD), a lymphotropic cytostatic substance, at 15 mg/kg dose killed 90% of mice treated previously with pertussis vaccine; in the non-vaccinated control group only 45% of the animals died. The vaccine in itself failed to exert a lethal effect. In combination with prolonged DAD treatment, 20% of the animals died. Examination of the lymphoid organs showed that animals with impaired lymphoid system exhibited an increased sensitivity to further damaging agents, independently whether they caused lymphoid atrophy or hypertrophy.

PARAPERTUSSIS — SEROEPIDEMIOLOGICAL STUDIES

G. NAUMANN and B. KÖHLER

*Institute of Medical Microbiology and Epidemiology, Wilhelm-Pieck-University,
Rostock, G.D.R.*

Using indirect immunofluorescence (IF) and Widal reaction (WR) a total of 420 random-selected sera taken in the district of Rostock were examined for the presence of antibodies against *Bordetella parapertussis*. Out of 420 sera investigated, 10 were collected from children probably infected with *B. pertussis*. Two other groups comprised 155 sera from infants and children and 255 sera from adults. Two-year-old children were positive in 51.1% with WR and in 74.8% with IF. A high rise in titres was observed in the 11 to 20 year age-group, the respective positive results were 75.0% and 97.2%. Maternal antibodies in infants sera detected up to 8 weeks after delivery, but not between 3 and 6 months of age. Ten children with clinical diagnosis of pertussis showed titres in the normal range.

IMMUNITY TO ENTEROTOXIC ESCHERICHIA COLI IN PIGS

L. PESTI and G. SEMJÉN

Veterinary Research Institute, Hungarian Academy of Sciences, Budapest

Anti-enterotoxigenic immunity was induced with an enterotoxin-rich *Escherichia coli* vaccine. (i) The licensed, adjuvanted vaccine preparation contained formalized heat labile (LT) and heat stable (ST) enterotoxins and

bacterial cells. Two 5 ml doses of vaccine were given intramuscularly to each sow 30 and 15 days before farrowing and two one ml doses were injected in 22 and 30-day-old pigs. (ii) To prove the effectiveness of the vaccine, paired sera and colostrum of pre- and postvaccinated sows were examined for the presence of LT-neutralizing antibodies. Sera of non-vaccinated pigs were also investigated to detect antibodies to LT. (iii) Detectable amounts of LT-neutralizing antibodies were present in sera and colostrum of pre-vaccinated sows and young pigs. The vaccination resulted in a significant rise of LT-neutralizing titre in sera and colostrum of sows. Anti-ST sera were obtained with hyperimmunization. (iv) In field trials, the anti-enterotoxigenic *E. coli* vaccine was effective in reducing the incidence of *E. coli* diarrhoea via the colostrum in piglets and via direct immunization in weaned pigs.

COMPARISON OF DIFFERENT METHODS FOR PRODUCING ESCHERICHIA COLI ENTEROTOXIN

G. SEMJÉN and É. CZIRÓK

*Veterinary Research Institute, Hungarian Academy of Sciences, Budapest and Pest County
Public Health Station, Budapest*

Heat labile enterotoxin (LT) production by standard enterotoxin-producing *Escherichia coli* strains was examined using three different media and various ways of preparation. Strains of human and animal origin not producing enterotoxin were used as controls. The presence of enterotoxin was assayed on ligated jejunal segments of rabbits. The most favourable results were obtained on tryptose agar; LT was also detectable when Smith's soft agar cultures were lysed by ultrasonic disintegration. On Sakazaki's medium modified by the authors (XMV) only one strain of porcine origin produced LT in demonstrable amounts.

LOCAL IMMUNITY AGAINST STREPTOCOCCUS PYOGENES INFECTION

J. FRANEK and V. KUBIN

*Institute of Experimental Medicine, Czechoslovak Academy of Sciences,
Prague, Czechoslovakia*

Patients with recurrent streptococcal infections of the upper respiratory tract were investigated by microbiological and immunological methods. There was no correlation between the IgG, IgM and IgA levels in serum and/or saliva, and the clinical status of impaired resistance. As demonstrated by the immunofluorescent method, the same is valid for the number of Ig-deter-

minant-bearing cells in human tonsils. The reaction of SIgA with streptococcal cells in saliva and on the surface of tonsils was investigated with heavy chain specific anti-IgA fluorescent antibody; there were substantial differences among individual patients. No significant correlation existed between the SIgA system activity and the number of bacteria adhering to the epithelial cells of the tonsils. The results indicate that the adherence-blocking mechanism of SIgA may explain only in part the role of local antibodies in the protection against infection.

ASSAY OF TETANUS ANTITOXIN BY PASSIVE HAEMAGGLUTINATION

H. GLATHE, CH. ZEISZLER and H. GIESECKE

Serum Co., Dresden and Regional Institute of Hygiene, Halle, G.D.R.

A passive haemagglutination method has been elaborated which allows the determination *in vitro* of 0.001 IU/ml tetanus antitoxin within two hr. Statistical comparison of the results showed a high degree of correlation with serum neutralization test at 0.99 level. The procedure is technically simple, and useful for a rapid determination of the immune status.

SOME CHEMICAL AND BIOLOGICAL PROPERTIES OF THE CAPSULE MATERIAL FROM STAPHYLOCOCCUS AUREUS STRAIN 1193/74

G. SELTMANN, W. WITTE, H. KÜHN and B. RICHTER

Institute of Experimental Epidemiology,¹ Wernigerode, G.D.R.

Though it is now generally accepted that the capsule material of *Staphylococcus aureus* increases the virulence, its importance in inducing antibacterial immunity is still controversial. This depends mainly on the lack of a biologically significant model of staphylococcal infection. In order to develop an antibacterial vaccine, some properties of the capsule material of *S. aureus* strain 1193/74 have been investigated. The material could be separated into numerous fractions with very similar chemical composition. All fractions contained uronic acid, galactose and amino acids in varying amounts. After injection into mice the partially purified material caused no significant antibacterial immunity. On the other hand, in the sera of these mice there was an increased titre of anticapsule antibodies as well as an increased opsonic index directed against *S. aureus* strain 1193/74.

PREPARATION AND EXAMINATION OF CARDIOLIPIN
FROM *TREPONEMA REITERI*

Z. RIEDL, M. SURJÁN and P. RICHTER

Institute for Serobacteriological Production and Research Human and National Institute of Hygiene, Budapest

From centrifuged and freeze-dried *Treponema reiteri*, cardiolipin was prepared in two different ways. After extraction with acetone and methanol, cardiolipin was produced by PANGBORN's modified procedure. The other method was VORBECK and MARINETTI's total-lipid extraction followed by column chromatography on Kieselgel-G. Both preparations gave characteristic cardiolipin reactions (ninhydrin negative, Dragendorff negative, ammonium molybdenate positive). While PANGBORN's method yielded an impure product as tested with thin-layer chromatography on Kieselgel-G, the other preparation was sufficiently pure. Antigens for serological reactions were prepared on the basis of the cardiolipin content of their solutions. The antigen prepared by PANGBORN's method corresponded in sensitivity and in specificity to the cardiolipin antigen made from beef-heart. The antigen prepared by the method of VORBECK and MARINETTI gave a specific product of low sensitivity.

ABOLITION OF ANTIGENIC COMPETITION BY IMMUNOSTIMULANTS
OF BACTERIAL ORIGIN

L. RÉTHY and L. BÁCSKAI

Institute for Serobacteriological Production and Research Human, Budapest

Correlation between the dose of administered immunostimulant and its preventing effect on antigenic competition were examined. As competing antigens, diphtheria and tetanus toxoids, as immunostimulants *Bordetella pertussis* organisms and Boivin-type extracts of shigellae were applied. Laboratory tests and field investigations in humans showed that (i) The application of the above-mentioned immunostimulants, depending on the timing of the introduction of different immunizing and/or adjuvanting substances, may completely abolish the competitive effect of antigens. (ii) Only simultaneously introduced immunostimulant can decrease or completely abolish the competition of antigens. Endotoxin introduced 2-3 days prior to or following the immunization with the competing antigens fails to prevent the immunological consequences of antigenic competition. (iii) The degree of abolishing effect shows a strict correlation with the amounts of endotoxin

administered simultaneously with the competing antigen system. The possible mechanism of action of the above phenomena and the cellular and/or sub-cellular events involved are discussed.

DETERMINATION OF THE MINIMUM ANTIGEN DOSE FOR COMPLETE PROTECTION OF MAN AGAINST TETANUS

L. HEGEDÜS, L. RÉTHY and L. BÁCSKAI

Institute for Serobacteriological Production and Research Human, Budapest

The laboratory effectiveness of adsorbed tetanus toxoids as determined by "one stimulant", "two stimulant" and "standard" methods is not acceptably correlated with results obtained in man. Consequently the mentioned laboratory methods are inadequate for defining the human immunizing capacity of vaccines containing tetanus toxoid. In a study of the correlation between results of toxin challenge test expressed in IU (Immunizing Unit) and immunization of man, the vaccines were tested against the International Tetanus Toxoid Reference Preparation and their own reference toxoid. The human immunizing capacity of tetanus toxoids was determined after two-shot immunization. As an arbitrary threshold value of complete human protection, 0.1 IU/ml serum was accepted. Monovalent tetanus toxoids exhibited 30–60 IU per immunizing dose in laboratory experiments; at the same time the vaccine provided only 50% protection in man. In case of combined vaccines (typhoid–tetanus, diphtheria–tetanus–pertussis) the laboratory effectiveness ranged between 150–600 IU per immunizing dose. All the above combined vaccines produced 0.1 IU/ml in man. The data suggest that the minimum immunizing dose of tetanus toxoid should be increased from 50 IU to 100 IU.

BIOLOGICAL STANDARDIZATION OF DIPHTHERIA, TETANUS, PERTUSSIS AND DIPHTHERIA–TETANUS–PERTUSSIS REFERENCE PREPARATIONS

J. ZSIDAI, Z. CSIZÉR, A. BENKŐ, N. NIEDERMAYER and L. HEGEDÜS

Institute for Serobacteriological Production and Research Human, Budapest

The relative potency of three reference vaccines prepared by the authors (freeze-dried adsorbed diphtheria toxoid, freeze-dried adsorbed tetanus toxoid and freeze-dried pertussis vaccine) was compared by active protection tests with the International Reference Preparations. The own reference vaccines contained 111.5 IU of diphtheria toxoid, 100.2 IU of tetanus toxoid and 90 IU of pertussis vaccine per ampoule. A freeze-dried adsorbed diphtheria–tetanus–

pertussis reference vaccine was also prepared. The potency of the components of this vaccine in comparison with the International Reference Preparations was 350 IU/ml, 250 IU/ml and 20.7 IU/ml, respectively. The dose-response functions of the single and combined reference vaccines were compared by analysis of variance.

ANTIGEN COMPETITION OF FOOT AND MOUTH DISEASE VACCINES OF "O₁", "A₅" AND "C" TYPES

F. SÓLYOM and F. CZELLENG

Phylaxia Veterinary Biologicals and Feedstuffs Co., Budapest

The aim of the studies was to establish the adequate rate of antigen components in bi- and trivalent vaccines. The antigen content of the vaccines has been expressed in complement-fixing units (CFU) and the efficacy has been confirmed by the E-index method. The results have shown that any form of the vaccine (mono-, bi- or trivalent) contains "O₁" to that of "A₅" or "C" antigens, the bivalent vaccine should contain about 50% and the trivalent vaccine 100% more "O₁" antigen than "A₅" or "C" components. Trivalent vaccines composed in this manner proved efficient in cattle experiments.

EXPERIMENTAL BRUCELLOSIS IN MICE WITH INHIBITED MACROPHAGE ACTIVITY

F. KEMENES

Institute of Epidemiology, University of Veterinary Sciences, Budapest

Mice injected subcutaneously with 0.3 ml Mastermix, an iron-dextran preparation, were infected intraperitoneally with 10^3 – 10^7 cells of *Brucella abortus*. Doses of 10^7 and 10^8 cells were lethal to almost all animals in one week, whereas 10^5 and 10^4 cells caused subacute brucellosis leading to death in 50–20% in 2–4 weeks. The control group survived even the highest doses, although the bacteria could be recovered from the enlarged spleen for 2–3 months.

SEPARATION OF SERUM IgG AND IgM GLOBULIN FRACTIONS
BY ADSORPTION ONTO $\text{Al}(\text{OH})_3$ GEL

E. SZÖLLŐSY, I. PAPP and I. SZABÓ

*Public Health Station, Szeged, First Department of Surgery and Second Department of Medicine,
University Medical School, Szeged*

Intratypic differentiation of enteroviruses and *Enterobacteriaceae* by $\text{Al}(\text{OH})_3$ gel adsorption proved an effective and quick procedure to characterize closely related enterovirus and bacterial strains. To investigate the use of this method for separating immunoglobulins, IgG and IgM fractions of poliovirus type 1 rabbit antiserum, *Escherichia coli* O55 rabbit antiserum and human sera passed through Sephadex G_{200} column were adsorbed onto $\text{Al}(\text{OH})_3$ gel and eluted with phosphate buffer of increasing molarity in one batch experiment. The amounts of eluted globulin fractions were measured by radioimmunoassay and acrylamide gel electrophoresis. The elution maxima of IgG and IgM varied according to the amount of $\text{Al}(\text{OH})_3$ gel used for adsorption: at 0.05 phosphate buffer molarity only IgG was eluted, whereas IgM appeared at 0.3 molarity.

ALTERATION OF HUMORAL IMMUNITY DURING PREGNANCY

S. PÁCSA, B. PEJTSIK and J. HADNAGY

*Institute of Microbiology and Department of Obstetrics and Gynaecology, University Medical
School, Pécs and County Hospital, Pécs*

Antibody titres in serum samples of 600 pregnant and 130 non-pregnant women were determined to rubellavirus, herpes simplex virus and *Toxoplasma gondii* by the haemagglutination-inhibition (rubellavirus) and the indirect immunofluorescence tests (herpes, toxoplasma). (i) Highest average titre was found in sera of women with spontaneous abortion. (ii) Antibody titre decreased as pregnancy progressed. (iii) Diminished humoral immunity is a transient phenomenon: immunity increased gradually post partum. (iv) Serological assessment of the role of infections in fetal malformations may be hampered by the impairment of humoral immunity during pregnancy.

FOLLOW-UP OF WOMEN WITH AND WITHOUT HERPESVIRUS-SPECIFIC ANTIGENS IN THEIR CERVICAL CELLS

S. PÁCSA, L. KUMMERLANDER, B. PEJTSIK, K. KROMMER and K. PÁLI

Institute of Microbiology and Department of Obstetrics and Gynaecology, University Medical School, Pécs and County Hospital, Pécs

The presence of herpes simplex virus-specific antigens was investigated by the indirect immunofluorescence technique in cervical cells of women (530 with normal cervical epithelium, 175 with mild disorders, 52 with dysplasia and 38 with invasive cervical carcinoma). Antigens were present in 9% of samples from women with normal cervical epithelium. Samples from women with mild disorders contained the antigens in 41%, from dysplasia patients in 61% and from invasive carcinoma patients in 94%. Testing three consecutive imprints of 68 antigen positive and 232 antigen negative women at six month intervals, showed that the herpesvirus-specific antigens persisted in the cervical cells through the one year period of the follow-up. The fact that herpes simplex virus-specific antigens were detected in normal cervical epithelium may support the initiating role of herpesvirus genitalis in the development of cervical cancer.

HUMORAL ANTIBODY LEVEL IN VARICELLA-ZOSTER INFECTIONS

P. GRÓF, J. HARANGI and S. PÁCSA

Institute of Microbiology, University Medical School, Pécs

To investigate the relation of humoral antibody level to primary varicella and to the clinical picture of zoster, the antibody level was determined by the indirect immunofluorescent method in 25 zoster patients at different intervals after onset. The titres were 1 : 5–1 : 5 in early (1–5-day) serum samples, 1 : 10–1 : 160 in 6–20-day and 1 : 20–1 : 80 in 21–53-day samples. In zoster patients treated with post-zoster serum 24 hr–7 days after onset, the specific antibody titre rose from 1 : 5 to 1 : 40. After 14–16 days the patients showed a remarkable clinical improvement and the antibody titre decreased gradually.

Interaction of pesticides and microorganisms

EFFECT OF MICROORGANISMS[ON PHYTOTOXICITY] OF HERBICIDES; INTERACTION OF SOME MICROORGANISMS WITH BETANAL

N. BALICKA and Z. KREZEL

Department of Microbiology, Agricultural Academy, Wroclaw, Poland

Betanal is used as a herbicide for sugar beet in foliar application after germination or thinning. Its active substance, phenmedipham, belongs to the carbamate group. A study was made of the microbial effect on Betanal phytotoxicity. The microorganisms were isolated from the rhizosphere and leaves of sugar beet and of some weeds. Mustard was used as a test plant: mustard seeds were saturated with the culture of a strain, and Betanal solution of 10–100 ppm was sprayed over the seedlings. The rate at which the plants died and the number of survivors was taken as the measure of microbial metabolite interaction with Betanal. Of 200 tested strains, 12 were chosen which produced the required changes. Ten of the strains mitigated the action of Betanal, and the other two increased it. These strains inhibited growth of the test plant, when used in proper concentration. The strains produce polysaccharide compounds, which are responsible for the effect. Therefore the interaction between microbes and Betanal is a resultant of two negative factors. The reducing mechanism of microorganisms on the phytotoxicity of Betanal is unclear; in some cases the herbicide decomposition might result in such an effect, though the course of experiment indicates that there might be another reason for it.

DECOMPOSITION OF DINITRO-O-CRESOL BY MICROORGANISMS

M. NEHÉZ, M. D. A. PÁLDY and A. SELYPES

Institute of Public Health and Epidemiology, University Medical School, Szeged

Environmental pollution by pesticides is one of the basic problems of public health. The quicker the decomposition of a pesticide, the more advantageous it is from hygienic aspects. From this point of view we investigated the decomposition by bacteria of dinitro-o-cresol (DNOC), the effective agent of the pesticide "Krezonit E". The bacteria applied were *Escherichia coli*, *Proteus vulgaris*, *Bacillus cereus* and *Bacillus subtilis*. All these were found to affect the decomposition of DNOC, especially at 37°C, their optimum temperature. The problem arose how these bacteria affected the toxicity of the pesticide during decomposition, and to what extent the metabolites are

toxic. Therefore by intraperitoneal administration to white mice we established the LD_{50} before and after the microbiological decomposition of DNOC by the method of LITCHFIELD and WILCOXON. DNOC content of the solution was evaluated by the PARKER method. During the microbiological decomposition of DNOC, no toxic metabolite was formed.

PESTICIDE-HUMUS COMPLEXES AND THEIR ROLE IN CROP CONTAMINATION

R. BARTHA

Rutgers University, New Brunswick, N. J., U.S.A.

Various pesticides and their degradation products were reported to form complexes with soil organic matter. The causative forces range from weak physical adsorption to the formation of extremely stable covalent bonds, with clear implications for residue persistence and residue monitoring. Using radiolabelled 3,4-dichloroaniline (DCA), a biodegradation product of the herbicide 3,4-dichloropropionanilide (propanil), we studied the hydrolyzable and non-hydrolyzable binding of DCA to whole soil and to isolated soil humic acid. The complex formation is a spontaneous chemical reaction occurring in both natural and sterilized soil. At the 5 ppm level, 80% of the applied DCA becomes unextractable by solvents within one day. As measured by $^{14}CO_2$ evolution, the half-life of the attached DCA in natural soil ranges between 1 and 2 years. This contrasts sharply with the less than one day half-life of propanil, the parent herbicide. Initially, about half of the unextractable radioactivity can be recovered as unchanged DCA following caustic or acid hydrolysis and steam distillation, but during prolonged incubation an increasing portion of the radiolabel shifts to the non-hydrolyzable form. For this reason, it is currently not feasible to assess the DCA burden of propanil-treated field soils, and an improved analytical technique is urgently needed. Based on bond stabilities and model reactions of DCA with monomeric constituents of humic acid we suggest that the hydrolyzable DCA residues are anils and anilinoquinones. Incorporation of the aromatic amino nitrogen into heterocyclic ring systems satisfactorily explains the non-hydrolyzable binding of DCA. Humus-degrading soil fungi attack DCA-humic acid complexes prepared *in vitro*. Two distinct modes of attack were noted, one resulting primarily in $^{14}CO_2$, the other mainly in free DCA and DCA attached to humic oligomers. The latter products can be taken up by plant roots and can contaminate the edible portion of the crop, e.g. the rice grain. Rice plants leaf-treated with radiolabelled propanil did not transmit detectable radioactivity to the grain, but untreated rice plants grown in propanil-treated soil did. The results of this preliminary experiment and a critical examination of data

from other research laboratories strongly suggest that the formation and subsequent biodegradation of pesticide-humus complexes constitute a heretofore unrecognized mechanism of crop contamination that may continue to be active long after the originally applied pesticide has become undetectable.

EFFECT OF HERBICIDES ON ANTIBIOTIC ACTIVITY OF BACTERIAL STRAINS ISOLATED FROM CUCUMBER PHYLLOSPHERE

Z. KREZEL and D. LESZCZYŃSKA

Department of Microbiology, Agricultural Academy, Wrocław, Poland

Antibiotic activity of 250 strains of saprophytic bacteria against 4 strains of phytopathogenic bacteria was examined. The phytopathogenic bacteria included two strains of *Pseudomonas lachrymans* and two of *Erwinia carotovora* (872 and 1008, from the Czechoslovak Collection of Microorganisms, Brno). Fifteen per cent of the tested strains exhibited antibiotic activity against *E. carotovora*, and 10% against *P. lachrymans*. The strain No. 160, which had been isolated with plant tests, was identified as *Bacillus subtilis* and exerted strong antibiotic action against the selected phytopathogenic strains. In micropot experiments, after treatment with a supernatant of strain 160 and infection with the phytopathogenic bacteria, the herbicides Venzar and CIPC modified the antibiotic action of the strain to some extent. The results point to higher activity of *B. subtilis* 160 in the presence of CIPC applied in 50 ppm doses. Higher and lower doses did not change the activity of that strain.

SPECIES DIVERSITY AND QUANTITY OF MICROSCOPIC FUNGI INFLUENCED BY HERBICIDE INTERACTIONS IN CHERNOZEM ECOSYSTEMS

Cs. DOBOLYI, Zs. PÁSZTOR and M. KECSKÉS

National Institute of Hygiene, Budapest and Research Institute for Soil Science and Agricultural Chemistry of the Hungarian Academy of Sciences, Budapest

The number of fungi in ecosystems of chernozem with forest residues changed under the effect of 0.01 and 0.1% 2,4-dichlorophenoxy acetic acid (2,4-D) according to the medium used. The decreasing effect of high doses (1%) of 2,4-D was very expressed (70%). The commercial preparation of 2,4-D-Na (Dikonirt) proved to be twice more toxic than the chemically pure 2,4-D and this was increased in the presence of 0.1% atrazine. The species diversity of fungi was also markedly changed, e.g. one strain of *Fusarium* sp. multiplied five times more than the control when affected by 1% 2,4-D,

and eight times more, when 0.1% atrazine too was added to the soil. The decomposition rate of 2,4-D and of Dikonirt used in agricultural practice as well as the effect of atrazine on their decomposition were measured by gas chromatography.

SOIL MICROORGANISMS AND HERBICIDE METABOLISM

J. SOBIESZCZANSKI, R. STEMPNIIEWICZ and G. MANIARA

Agricultural University Institute of Food Science and Technology and Department of Technical Microbiology, Wrocław, Poland

After penetrating the soil, the herbicides act not only as weed-killers, but as physiologically active compounds they exert various effects on the environment, and are affected by natural systems and physico-chemical environmental factors. A particular role in these processes is played by soil microorganisms. In the present studies plants in degraded black soil, sand or claysand were treated annually for many years with high doses of triazin or urea compounds. The results revealed by microbiological methods, thin layer chromatography, column elution chromatography and gas chromatography indicate that the duration of herbicide action in the soil depends mostly on the chemical structure of the herbicide. In the course of herbicide metabolism in the soil, a few or several new compounds can be detected which are lacking from the control, untreated soil. The R_F of the compounds varies according to the levels of the soil.

EFFECT OF A FEW HERBICIDES ON THE MICROBIOLOGICAL DECOMPOSITION OF CORN-STALK

B. TÓTH

University of Agricultural Sciences, Keszthely, Hungary

The carbon-dioxide producing capacity of brown forest soil collected in the neighbourhood of Keszthely was measured under laboratory conditions, using various doses of Ramrod, Adtikon, Afalon and Hungarian K-64 herbicides and chemical fertilizers, with and without corn-stalks. The herbicides hindered CO_2 production considerably if applied at 100–400 times higher than the usual concentrations. This inhibiting effect was considerably reduced by adding an adequate dose of nutritive material supplement (100–500 mg N/200 g soil). The doses of Hungarian K-64 and Adtikon stimulated the CO_2 production of soil in the first 12–14 weeks of the 20-week experimental period. Ramrod and Afalon had no appreciable effect on the quantity of CO_2 produced by the decomposing corn-stalks.

BIODEGRADATION OF PIGMENTED FUNGAL SPORES IN NATURAL SOILS

K. M. OLD and Z. A. PATRICK

Biological Sciences Department, Dundee, Scotland and Botany Department, University of Toronto, Toronto, Canada

Pigmented fungal propagules are well-known to be markedly resistant to biodegradation in soils. This is particularly important in the case of plant pathogenic fungi which survive in a dormant state in the soil in the absence of a susceptible host plant. A biological agent present in many soils is able to initiate lysis and death of conidia of *Cochliobolus sativus*, *Thielaviopsis basicola* and other pigmented species by perforating the spore cell wall. The perforating agent(s) have not been isolated and identified so far. Experiments using filter membranes with several pore sizes have demonstrated that the agent is able to penetrate membranes of 1.0 μ and 5.0 μ pore diameter, but not those of 0.2 μ and 0.6 μ pore diameter. Direct observation of spores by light and electron microscopy indicated that bacteria and possibly protozoa may be responsible for perforation of spores. Once the outer pigmented cell wall layer is penetrated, a wide range of soil microorganisms colonize the spore wall and cell lumina, and the spores are destroyed. Perforation-lysis of spores occurs in many natural soils and appears to be a biological control mechanism that may exert an influence on the population level of those plant pathogenic fungi that survive in the soil by means of pigmented propagules.

INTERACTIONS OF LINURON AND SOIL MICROORGANISMS

T. CSERHÁTI and M. KECSKÉS

Research Institute for Plant Protection, and Research Institute for Soil Science and Agricultural Chemistry of the Hungarian Academy of Sciences, Budapest

The decomposition of 10 ppm linuron (N-3,4-dichlorophenyl-N'-methoxy-N'-methylurea) was investigated in sterilized and intact chernozem with forest residues soil at 10, 18, 26 and 37°C. The ratio of the microbiological decomposition was negligible (74–55%). The activation energy for chemical decomposition was 19.4, for microbiological decomposition 13.3 kcal/mol. The rate constants measured in sterilized soil and in aqueous buffer solution having the same pH did not differ significantly. The decomposition of linuron (5 ppm) and the change of the "total" number of microbes were studied at 26°C in this soil and brown forest soil ecosystems affected by TMTD and 2.5% Hg-content Falisan fungicides (30 ppm), gamma-BHC, diazinon zoocides (30 ppm), gamma-BHC and TMTD combination (15 + 15 ppm) as well as by propachlor, simazine, methoprotthyne, Camparol 1803: promethryne + sima-

zine herbicides (5 ppm). The decomposition rate of linuron was decreased markedly by the insecticides and gamma-BHC + TMTD combination and especially by Falisan on the first week after addition of pesticides to both soils. The applied doses of herbicides did not influence the decomposition of linuron. There was no close connection between the change in the "total" number of microbes and the decomposition rate of linuron. The linuron residues were measured by ECD gas chromatography.

EFFECT OF BIOCIDES ON THE MICROBIOLOGICAL STABILITY OF PLASTICS

I. GYARMATI, K. CSISZÁR, Z. WOLKÓBER and P. FARKAS

Institute of Microbiology, Semmelweis University Medical School, Budapest and Research Institute of the Hungarian Post Office and Telecommunication Works BHG, Budapest...

Biocides are additives added to plastics during manufacturing in order to increase their microbiological resistance by rendering them bactericide or fungicide. Such additives are boron and copper compounds, organic tin, arsenic, phenol derivatives and quaternary amines. We have examined 16 biocides that were present in soft PVC foils of different chemical composition, using *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Candida albicans* as biological agents. The growth inhibitory action of these biocides concerned primarily Gram-positive bacteria and *C. albicans*. No measurable bactericide effect could be detected with respect to the Gram-negative bacteria. Stanicid F., Preventol A-3-Preventol K-1, Barozid and copper-oxyquinoline biocides proved the most effective. The effective action of biocides depends on the chemical composition of plastics. In the individual additives of plastics provide suitable culture media for bacterial proliferation, the microbes attack the plastics irrespective of the presence of the above biocides.

EFFECT OF HERBICIDES ON QUANTITATIVE CHANGES OF SOIL BACTERIA

B. HELMECZI

Soil Science and Microbiological Department of the University of Agricultural Sciences, Debrecen

The effect of ten different herbicides (Afalon 20 EC, Aktikon 90 WP, Eptan SH 20, Karbamic + Satecid 65 WP, Kartex A, Kartex B, Propaklór 40 EC, Satecid 65 WP, Sutan 80 EC, Terbutrin 50 EC) and their combinations was investigated on the change of aerobic N-fixing, aerobic cellulose decomposing, ammonifying and nitrifying soil bacteria in small plot field experi-

ments conducted in latin block arrangement on chernozem soil using maize as test plant. The number of bacteria was determined from samples collected in spring, summer and autumn, with POCHON's modified method. (i) Among four physiological groups nitrifying bacteria were the most sensitive to herbicides. The inhibitory effect of treatments changed 5–32% in 1974, and 14–40% in 1975. (ii) Least sensitive were the aerobic cellulose decomposing bacteria.

Virology

IMMUNOFLUORESCENCE ANALYSIS OF ADENOVIRUS PROTEIN

N. S. DYACHENKO, L. N. NOSACH, N. P. VANTSAK and L. V. TOKARCHUK

The D. K. Zabolotny Institute of Microbiology and Virology, Academy of Sciences of the Ukrainian S.S.R., Kiev, U.S.S.R.

The dynamics of appearance, intensity of accumulation and peculiarities of distribution in the cells of early and capsid adenovirus proteins have been studied by the indirect fluorescent antibody technique with monospecific sera and cytofluorometry. Early antigen of type 1 adenovirus appears 6 hr after infection in 4.5% of cells as little granules in the nucleus and as diffuse luminescence in the perinuclear zone of some cells and reaches the maximum 12 hr after infection. The sequence of appearance of capsid proteins was: fibre, followed in one hour by hexone (14th–25th hr) and finally by pentone (16th–17th hr). The same sequence is observed in quantitative analysis of protein accumulation, but a reliable increase in average intensity is recorded by the fluorescence cytofluorometric method only 2–4 hr after their appearance. Capsid proteins show a similar localization. First they are observed as granules in the nucleus; the cytoplasm fluorescence is characteristic for hexone and, to a less extent, for pentone. Later the protein appears on DNA-containing inclusions and as aggregations under the nuclear membrane in the inclusion surrounding a zone devoid of DNA. The synthesis of capsid proteins and hexone in particular is a thermosensitive process hindered during incubation of the infected cells, at 42°C its fluorescence being two times less intense than in cells incubated at optimum temperature. Hexones within the inclusion and abundant protein aggregations are localized so that their group-specific determinant is in metatopic position and is accessible to antibodies.

PROPERTIES OF ADENOVIRUS HEXONES

É. ÁDÁM and I. NÁSZ

Institute of Microbiology, Semmelweis University Medical School, Budapest

Hexones incorporated in the intact capsid of adenovirus type 1 and those found in soluble form in infected cell cultures were studied by electron microscopy. The virions and soluble hexones were separated and purified by ultracentrifugation, anion-exchange chromatography and gel filtration. Two different preparation procedures, viz., negative staining and the freeze-fracturing technique, were applied. The dimensions of the soluble hexones have been determined, and it was found that the capsomer consists of three polypeptide molecules. The homology of the purified hexone preparation was controlled by polyacrylamide gel electrophoresis, electron microscopy and immunological techniques. From the hydrolysate of the pure hexone preparation, the amino acid composition of adenovirus type 1 hexone has been established.

HAEMAGGLUTININATING PROPERTIES OF A PARVOVIRUS-CONTAMINATED
TYPE 12 ADENOVIRUS STRAIN

A. LENGYEL, M. TÓTH, M. BAKAI, I. BÉLÁDI and I. NÁSZ

Institute of Microbiology, Semmelweis University Medical School, Budapest and Institute of Microbiology, University Medical School, Szeged

One of the authors' human adenovirus type 12 strains proved to be contaminated by parvovirus when examined by electron microscopy. The strain agglutinated human, guinea-pig, and, to a lesser degree, ovine erythrocytes. To identify the parvovirus strain, neutralization and haemagglutination inhibition tests were carried out. The buoyant density of the parvovirus was determined and the virus purified by ultracentrifugation and gel filtration. In both instances two distinct kinds of haemagglutinin were detected, a virion-bound and a soluble one. The latter was also examined by anion-exchange chromatography. The conditions and optimum of virus adsorption to, and elution from, blood cells were established. Attempts were made to characterize the parvovirus-binding erythrocyte receptors and an adsorption-elution method was evolved to separate the parvovirus from the adenovirus. The interferon-inducing ability of the purified adenovirus-free parvovirus was examined in chicken cells.

INTERACTION IN REPLICATION OF GOOSE PARVOVIRUS STRAIN B
AND DUCK PLAGUE HERPESVIRUS

J. KISÁRY

Veterinary Research Institute, Hungarian Academy of Sciences, Budapest

The goose parvovirus strain B belongs to the first genus of the Parvovirus family. It needs no helper virus for replication, but the replicative processes are dependent on the mitotic cycle of the infected cells. In infected confluent monolayer of goose fibroblast cells only a low virus yield can be expected. Simultaneous infection of non-mitotic cells with parvovirus and herpesvirus resulted in nearly the same parvovirus yield as infection of mitotic cells with parvovirus alone. No similar potentiating effect of herpesvirus replication was significantly inhibited by parvovirus, in both mitotic and non-mitotic cells.

RECOVERY OF LATENT HERPESVIRUS FROM THE EXPLANTS OF RABBIT
REGIONAL SENSORY GANGLIA

J. RAJCÁNI, A. SABÓ, H. LIBIKOVÁ and F. CIAMPOR

Institute of Virology, Slovak Academy of Sciences, Bratislava, Czechoslovakia

More than 50 albino rabbits were inoculated into the right cornea with 10^7 p.f.u. of the Kupka strain of human herpesvirus type 1 (HHV 1). Between 1–9 months postinfection, both Gasserian ganglia, both trigeminal nerve trunks and pieces of the brain stem and right cornea were explanted. Activation of "latent" HHV 1 was found mainly in the homolateral ganglion, but also in the opposite one. The presence of virus in explants was assessed by infectivity titrations and morphological methods (immunofluorescence, electron microscopy, autohistoradiography). Within 24–72 hr prior to the appearance of HHV 1 in the medium, infectious virus was detected in a given explant at a ratio 1 to 10^4 cells. Alternatively, the presence of virus antigen in a few neurones and non-neural cells was revealed by immunofluorescence in serial sections. When ganglia were explanted in the presence of antiserum, the rate of virus recovery was at least two times lower as compared with the number of virus-yielding explants kept in a medium without antiserum.

EFFECT OF HERPESVIRUS INFECTION ON THE MITOTIC INDEX
AND CYTOGENETIC CHARACTERISTICS OF HUMAN EMBRYONIC
LUNG (R) CELLS

T. L. VARADINOVA, A. MINCHEVA, P. ANDONOV and I. BRADVAROVA

*Faculty of Biology, University of Sofia and Department of Virology CIPD, Medical Academy,
Sofia, Bulgaria*

Three antigenically distinct strains of herpes simplex virus (HSV) type 1 strain, RT; type 2 strain, DD and an intermediary strain, MB 1) were investigated for their influence on the mitotic index (MI) and the cytogenetic characteristics of R cells. The MI increased in cells infected by the MB 1 or DD strain, mainly due to a numerical increase in anomalous forms (metaphase with lagging chromosomes, anaphase with chromosome bridges). In MB 1 infected cells K metaphase (42% of all pathological forms), in DD-infected cells multipolar mitoses (29%), were the most frequent anomalies. In cells infected by the RT strain, the MI was decreasing, a phenomenon suggestive of a pronounced chromosome damage. The postinfection chromosome damage seemed to be progressing with time. Chromatid and isochromatid changes as well as chromosome breakages and gaps were also seen. The latter occurred more frequently in groups A and B, than in groups C and D, of chromosomes. It is suggested that the chromosome structure is less affected by the type 2 strains of HSV than by type 1 and intermediary strains.

VIROLOGICAL STUDIES ON PATIENTS WITH RECURRENT ORAL
MUCOUS MEMBRANE ALTERATIONS

J. HORVÁTH, P. DÁN, G. KULCSÁR, K. SALLAY, I. NÁSZ and P. GECK

*Institute of Microbiology and Department of Oral Surgery, Semmelweis University Medical
School, Budapest*

Virological studies were carried out on patients suffering from chronic recurrent ulcerous-vesiculous disease of the oral mucosa. An attempt was made to isolate virus from the saliva of 49 patients, on established cell lines. In 13 instances, herpes simplex virus (HSV) was isolated. Cells obtained from local lesions, and those from the intact oral mucosa during the symptom-free period, indicated the presence of HSV antigens on 20 occasions, and in 18 cases adenoviral antigens, by the help of immunofluorescence (IF). (The total number of cases examined by IF was 63.) Cells taken from the oral mucosa of 12 patients were examined electron microscopically, and in 6 instances virus particles were detected in both pathological and apparently intact cells.

SEARCH FOR ANTIBODIES TO HERPES SIMPLEX VIRUS TYPE 1
AND TYPE 2 IN PATIENTS' SERA

M. SIMON

Hungarian Army Medical Corps, Budapest

Serum samples from 150 patients suspect of herpes simplex virus (HSV) infection were tested for virus-specific antibodies with several serological tests. Demonstration of HSV-specific antibodies of the IgA and IgM types by immunofluorescence (IF) proved to be of the greatest diagnostic value. When both of these tests were performed, a single serum sample was often sufficient for the diagnosis of acute HSV infection, and also recurrent infections could be revealed in this way. The serotype of the virus can be demonstrated by the so-called staphylococcus rosette-formation test, a method evolved by the author. The common serological tests, viz., neutralization, passive haemagglutination, complement fixation, demonstration of IgG-type antibodies by IF, and the membrane IF method, all proved to be of inferior value. Evaluation of the results of these tests always needs simultaneous testing of paired sera, and even so mainly primary HSV infections can be diagnosed.

STIMULATION WITH CMV OF THE CELLULAR (SEMICONSERVATIVE)
DNA SYNTHESIS AND OF THE REPAIR MECHANISM OF THE CELL

I. BOLDOGH, É. GÖNCZÖL, L. GARTNER and L. VÁCZI

Institute of Microbiology, University Medical School, Debrecen

The stimulating effect of human CMV on cellular DNA synthesis and repair mechanism as well as on the mitotic index of cell cultures was demonstrated by the authors in both permissive (human embryonic fibroblast) and non-permissive (murine embryonic fibroblast) cells. The semiconservative replication of cells induced in serum-free medium increased 3 to 11-fold as compared to uninfected cells. In CMV infected cells injured by IUdR the DNA after repair was accelerated 2 to 5 fold. Owing to the semiconservative DNA replication, the mitotic index of the CMV-infected cells was elevated. Since in cells pretreated with partially inactivated CMV only the nuclear antigen demonstrable by the anti-complement immunofluorescence test was revealed during the observation period, it is suggested that stimulation of cellular macromolecular synthesis may be a very early viral function.

CYTOMEGALOVIRUS AND RUBELLA VIRUS ANTIBODY DETERMINATIONS
IN INFANTS WITH CONGENITAL MALFORMATION

Zs. KÓSA, I. MEZEY, G. NAGY and M. SIMON

National Institute of Hygiene, Budapest and Hungarian Army Medical Corps, Budapest

In 1974 and 1975, blood samples taken from 179 infants with congenital malformation were tested for cytomegalovirus (CMV) complement-fixing (CF), rubella haemagglutination-inhibiting (RHI) as well as CMV- and rubella-specific IgM antibodies. In 106 cases the CMV CF and RHI antibody levels of the mothers were also determined. Seven of 60 infants tested within 2 weeks after birth had IgM-type CMV-specific and high-titre CMV CF antibodies, suggesting that they had experienced an intrauterine CMV infection. The diagnosis of congenital rubella syndrome was ascertained by detection of rubella-specific IgM antibodies in blood samples of 3 newborns, and by demonstration of persistence of high-titre RHI antibodies for more than 4 months after birth in 10 cases. A titre increase in CMV CF antibodies combined with appearance of CMV-specific IgM antibodies could also be found in 4 of the latter 10 cases shortly after birth. Twenty-one infants were over 6 months of age when their first blood samples were obtained. The geometric mean titre of CMV CF antibodies was significantly higher in this group of infants than that in the group of matched healthy controls.

DEMONSTRATION IN THE PERIPHERAL BLOOD OF LYMPHOCYTES
CONTAINING EPSTEIN-BARR-VIRUS-ASSOCIATED NUCLEAR ANTIGEN (EBNA)

L. GERGELY, J. CZEGLÉDY, A. SZÁLKA, L. VÁCZI and E. BERÉNYI

Institute of Microbiology, University Medical School, Debrecen, Department of Pneumology, Semmelweis University Medical School, Budapest and Central Hospital for Infectious Diseases, Budapest

EBNA-positive cells were searched for in appropriately purified B lymphocytes obtained from peripheral blood. During the acute phase of Paul-Bunnell-positive infectious mononucleosis, 0.1 to 0.2% of the cells were EBNA-positive. The EBNA-positive cells disappeared during convalescence and did not reappear later. Circulating EBNA-positive cells could not be demonstrated in Hodgkin and SLE patients having high EBV antibodies. In three cases unsuccessful attempts were made to demonstrate EBNA-positive cells in Hodgkin lymph nodes.

SERODIAGNOSTICS OF INFECTIOUS MONONUCLEOSIS

M. KOLLER, Zs. KÓSA and M. SIMON

National Institute of Hygiene, Budapest and Hungarian Army Medical Corps, Budapest

Demonstration of anti-EBV IgM antibodies is an important tool in the diagnostics of infectious mononucleosis (IM). However, in heterophilic-antibody-negative cases, evaluation of the reaction is sometimes disturbed by the presence of anti-IgG antibodies of the IgM type and antibodies giving cross reactions with cytomegalovirus (CMV). Seventy-five IM patients were tested for further EBV-specific antibodies, viz., anti-EBNA and anti-EBV IgA antibodies, as well as for anti-CMV CF, IgM and IgA antibodies. The latter appeared in 3/4 of the EBV infections. In dubious cases, the presence of virus-specific IgA antibodies is a more specific indicator of EBV infection than of the IgM type antibodies. The presence of heterophilic antibody proves in itself the aetiological role of EBV and in these cases other serological investigations are unnecessary. The aetiological diagnosis of Paul-Bunnell-negative cases requires, however, the parallel use of several virus-specific serological tests.

HEPATITIS ANTIGENS IN SERUM AND STOOL SAMPLES OF PATIENTS WITH HEPATITIS

Á. PÁLFI, B. LÁSZLÓ, L. TELEGDY, Ö. POHL and I. HOLLÓS

National Institute of Hygiene, Budapest and Central Hospital for Infectious Diseases, Budapest

Serial serum and faecal samples from adult patients with the diagnosis of acute or chronic hepatitis have been examined for the presence of HB_sAg and IH_xAg by reverse passive haemagglutination and immunodiffusion tests. HB_sAg proved to be present in sera of half of patients with acute, and of a quarter of those with chronic, hepatitis. This antigen was demonstrable in the stools of the former group in 31% and in that of the latter group in 19%. IH_xAg could be detected in sera of 8% of patients with acute, and in 9% of those with chronic illness. In 12% of the acute and 20% of the chronic cases this antigen was excreted with the stools. Control healthy adults proved to be positive in 2% for HB_sAg and in 9% for IH_xAg. The high frequency of HB_sAg in the stools of hepatitis patients suggests that the non-parenteral route may play an important role in spreading the infection.

IMMUNOLOGICAL RESPONSE OF HEPATITIS PATIENTS TO VIRAL ANTIGENS

P. DÁN, I. HOLLÓS, J. HORVÁTH, G. KULCSÁR, A. MATKOVICS, I. NÁSZ and
G. HALMOSDI

Institute of Microbiology, Semmelweis University Medical School, Budapest, Municipal Hospital of Újpest, Budapest, National Institute of Hygiene, Budapest and Postgraduate School of Medicine, Budapest

In 53 patients with viral hepatitis (44 HB_sAg-positive and 9 non-type-B cases) and 6 healthy HB_sAg carriers the humoral immune response was examined by the complement-fixation (CF) test. Anti HB_s was found in 2% of the patients, antibodies to influenza A, herpes simplex and adenovirus in 8, 10 and 21%, respectively. The serum sample was anticomplementary in 22%. All the antibodies were present in low titres (1 : 2 – 1 : 8). None of the healthy HB_sAg carriers had any of these antibodies. Cellular immunity was examined by the lymphocyte transformation test. Increased sensitization against herpes simplex, influenza and/or purified HB_sAg was found in more than half of the cases. Among the healthy carriers only one showed an increased rate of blastic transformation when tested with herpesvirus antigen. The patients showing increased blastic transformation with herpesvirus had no demonstrable CF antibodies to this virus. The phytohaemagglutinin index of the patients who recovered ranged between 40% and 80%. It is suggested that not only the T system, but also the B system can be altered in the course of viral hepatitis.

HETEROGENEITY OF THE "d" DETERMINANT OF HEPATITIS B
SURFACE ANTIGEN

G. HALMOSDI

Postgraduate School of Medicine, Budapest

Three hundred and twenty HB_s antigens were subtyped for *d* and *y*. Of the positive blood donors, 96 had determinant *d*, 54 had determinant *y* and 3 had both *d* and *y*. In the patients with acute hepatitis, 50 were *ad*, 62 *ay* and 8 *ady*. In chronic hepatitis 25 were *ad* and 22 *ay*. Subtype *ad* did not predispose to chronic carrier state. Subtype *ay* showed a slowly-increasing percentage in acute hepatitis. A new determinant was observed between two *ad* antigens. This could not be explained by the *w* and *r* determinants. Out of 98 *ad* antigens 97 possessed the new determinant. It is concluded that the *d* determinant was heterogeneous and included at least two subdeterminants: *d*₁ and *d*₂. In 7 of 55 *ay* sera, *d*₂ determinant was detected. The new subtype: *ad*₂*y* is believed to be an intermediate form between *ad* and *ay*. It may prove identical with one of the newly-discovered determinants of HB_sAg.

SPECIFICITY OF THE EFFECT OF HB_sAg ON NICOTIANA SYLVESTRIS

I. HOLLÓS, Á. PÁLFI and B. GYÖRGY

National Institute of Hygiene, Budapest and Research Institute of Horticulture, Budapest

In agreement with literary data leaves of *Nicotiana sylvestris* exhibited lesions after injection of sera with high HB_sAg titre. The antigen could not, however, be detected in the leaves 24 hr after administration. Essentially similar lesions of *N. sylvestris* leaves could be produced in certain cases with HB_sAg-negative sera or saline. When 5×10^6 p.f.u. vaccinia virus had been injected into leaves, sharply circumscribed lesions appeared rapidly and reisolation of vaccinia virus was successful at 24 hr but failed 48 hr after inoculation. Leaves inoculated with vaccinia virus or saline equally contained a substance ("antiviral factor") which inhibited the multiplication of vaccinia virus on the chorioallantoic membrane of embryonated eggs. The inhibitor is not identical with nicotine, since similar results were obtained with extracts of *Phaseolus* sp. leaves inoculated with vaccinia virus. It is assumed that the lesions described are caused by the non-specific defence mechanism of the plant.

INVASIVENESS AND PERSISTENCE OF HUMAN HONG KONG INFLUENZA
A VIRUS VARIANTS IN CHICKENS

J. ROMVÁRY

Veterinary Research Institute, Hungarian Academy of Sciences, Budapest

Strain influenza A (H3N2) Hungary/1/73, closely related to England 42/72, was carried over 8 alternative passages in chicks and embryonated hen's eggs. With the line thus obtained (1/73/p8), and also with the original strain (1/73), chickens were inoculated intraorbitally and into the pharynx. The virus could be reisolated from sinus samples 11–25 days post inoculation. Reisolation of virus from chickens kept for 11–35 days in a common room with chickens that had been inoculated with 1/73 or 1/73/p8 7–22 days earlier was successful in 21–37% of the cases. From the contacts of chickens infected by 1/73, the virus could be reisolated up to days 22–44, whereas 1/73/p8 persisted for 48–54 days. Chickens infected by 1/73/p8 were reinoculated with the same virus on the 80th day after the primary inoculation. From the reinoculated birds, the virus could be reisolated on days 10 and 12 post infection, not only from the sinus, but also from the trachea, the lungs and the spleen. It is concluded that experimental adaptation of a human strain of influenza A virus resulted in an increase of its invasiveness for, and a prolonged persistence in, chickens.

STANDARDIZATION OF THE NEWCASTLE DISEASE
HAEMAGGLUTINATION INHIBITION TEST

B. TÓTH and V. P. JUHÁSZ

Phylaxia Veterinary Biologicals and Foodstuffs Co., Budapest

The HI titre of 61 individual sera originating from ND-vaccinated birds were determined against 8, 4 and 2 haemagglutinating (HA) units of the LaSota virus strain. The geometric mean for the HI titres against 8, 4 and 2 HA units was 23.75 , 24.59 and 24.85 , respectively. From the average values for the three groups, a regression equation has been calculated; it meets the statistical requirements. The values found in two groups show a good agreement with the empirical curve at the levels of 4 and 8 units, while at 2 units the variation is of higher degree. As the relationship is not fully linear, it is not justified to express the antibody content of sera in HI units. The HI titres of 20 individual sera were determined in tubes, and on WHO and TAKÁTSY's plates in parallel. The titre values in tubes and in WHO plates showed a close correlation. A similar tendency was observed with TAKÁTSY's micromethod, but the variation was higher. To set right the HI system, the use of a standard serum is highly recommended.

A MICROMETHOD FOR THE DETERMINATION OF INFLUENZA
VIRUS NEURAMINIDASE AND ANTI-NEURAMINIDASE

GY. TAKÁTSY and K. BARB

National Institute of Hygiene, Budapest

A micro-modification of AMINOFF and WARREN's method has been developed. The test is performed in wells of special plexiglass trays and the reaction mixture is heated electrically to keep the reading-temperature. A spectrophotometer is not required. The accuracy of the quantitative anti-neuraminidase test satisfied all requirements. The required amounts of reagents are about one tenth of those necessary in the original test. This is of special importance as regards the expensiveness of the substrate. The reaction in this simple form is suitable for wide-scale screening tests, even in moderately equipped laboratories.

MEASLES IMMUNOPROPHYLAXIS IN THE GDR

G. STARKE and E. GERIKE

Institute for Applied Virology, Berlin, G.D.R.

For the elimination of measles by immunization with live virus vaccine, the following points should be included in the immunization programme. (i) Vaccine must be available. (ii) The age specific morbidity distribution has to be studied before beginning with immunoprophylaxis. (iii) The immunization rates of the susceptible age groups should attain about 95%. (iv) Vaccinations should be carried out continuously and not be limited to campaigns of short duration. (v) The immune status of the population should be monitored by epidemiological-statistical reports and sero-epidemiological surveillance. (vi) The necessity of revaccination and cases of measles in spite of previous vaccination should be studied in order to evaluate the effectiveness of measles immunoprophylaxis. Experiences and results obtained from 1967 through 1975 are reported in detail.

INTRACEREBRAL INOCULATION OF DIFFERENT MUMPS VIRUS STRAINS
INTO NEWBORN HAMSTERS AS A METHOD OF PRE-TESTING
FOR THEIR NEUROPATHOGENIC PROPERTY

P. HLINAK, M. HILGENFELD, G. POLLEX and G. STARKE

Institute for Applied Virology, Berlin, G.D.R.

In order to reduce the number of monkeys in neurovirulence tests of mumps virus vaccine candidate strains, small laboratory animals were used for pre-testing these strains. Newborn hamsters were inoculated intracerebrally with different mumps virus strains. Different rates of resulting hydrocephaly were due to inflammation of the ventricular system. In addition to growth retardation, incoordination of movements and spastic paresis were observed. Certain pathohistological similarities were noted with changes seen in the CNS of hamsters following intracerebral inoculation and of monkeys following intrathalamic injection of mumps virus.

IMMUNOLOGICAL DEMONSTRATION OF ONCORNAVIRUS GENOME IN LEUCOCYTES OF MYELOID LEUKAEMIC AND PRELEUKAEMIC PATIENTS

F. D. TÓTH, J. JÁKÓ, L. VÁCZI, K. RÁK and I. RÉDAI

*Institute of Microbiology and Second Department of Medicine,
University Medical School, Debrecen*

Leucocytes from 10 patients with acute myelogenous leukaemia (AML) 15 patients with chronic myelogenous leukaemia (CML), 6 preleukaemic subjects and 20 healthy controls were tested for oncornavirus antigens by indirect immunofluorescence. Heterologous antiserum to LPV, viz., a virus isolated from human haemocytoblastosis, and antisera to the p30 antigens of simian, feline and murine oncornaviruses were used. All the AML leucocyte samples taken in an advanced stage of the disease contained LPV-specific antigen as well as simian and murine p30-specific antigens. During partial remission the murine, whereas during complete remission both the murine and simian p30 specificities, were lost. LPV and simian p30-specific antigen were demonstrated in 5 of the 15 CML patients. The antigen-positive patients were in the preblastic or blastic stage or could be kept in equilibrium with some difficulties by chemotherapy. Of the 6 preleukaemic patients three, viz., one with sideroblastic anaemia developing into erythroleukaemia, another with thrombocytopenia and the third with leukaemoid reaction, were found to be positive for LPV and simian p30.

THE PROGNOSTIC IMPORTANCE OF PLASMA cAMP LEVEL IN RAUSCHER LEUKAEMIA

A. RÉTHY, M. HALMY, F. D. TÓTH, M. KÁSA and L. VÁCZI

Institute of Microbiology, University Medical School, Debrecen

We have investigated the cAMP metabolism in tumour cells, and the cAMP level of plasma in inbred mice, infected by Rauscher leukaemia virus. In Balb/c erythroblasts (homogenates) the ratio adenylate cyclase/cAMP phosphodiesterase decreased with tumour progression. For the decrease, the loss of adenylate cyclase activity in the surface membrane was mainly responsible. In the same period the hormone-sensitivity of membrane-bound adenylate cyclase was decreasing considerably. The cAMP content of leukaemic plasma decreased dramatically in the highly susceptible Balb/c mice before tumour progression, and in the DRA mice, prior to the lethal exacerbation. The plasma cAMP level did not change in resistant C57B1 mice. The results strongly suggest that the cAMP level of leukaemic plasma may have a prognostic importance.

FATTY ACID COMPONENTS OF PHOSPHOLIPIDS IN RAUSCHER
LEUKAEMIA CELLS

I. RÉDAI, P. BIACS, J. KISS and F. D. TÓTH

*Institute of Microbiology, University Medical School, Debrecen and Department of Agricultura
Technology, Technical University, Budapest*

The phospholipids obtained from Rauscher-virus-transformed cells contain the same fatty acid components as those of normal mouse cells. In the tumour cells the fatty acid components of phospholipids show a specific distribution closely similar to that found in normal cells. The absolute quantity of the unsaturated fatty acid components of phospholipids of tumour origin is consistently increased, whereas the ratio of unsaturated to saturated fatty acid components depends on the other components of the phospholipid. In the tumour cell phospholipids C₁₈ fatty acids are reduced in quantity, whereas the highly unsaturated fatty acids containing 20 or more C atoms and the unsaturated fatty acids of more than 20 C atoms are increased in amount. It is supposed that phospholipid synthesis is affected in Rauscher leukaemia cells.

PRIMARY TUMOURS INDUCED BY MC29 VIRUS IN TURKEYS

Zs. SCHAFF, M. TÁLAS, I. STÖGER, I. FÖLDES and K. LAPIS

*First Institute of Pathology, Semmelweis University Medical School, Budapest and Micro-
biological Research Group of the Hungarian Academy of Sciences, Budapest*

Twelve to 14 days after intravenous injection of MC29 virus ($10^{5.5}$ LD₅₀) into one-day-old turkeys, hepatic tumours leading to death were observed in all the inoculated birds. The tumour proved to be hepatomas by light and electron microscopy: large amounts of C-type particles were found between tumour cells and budding from the cell-membranes. C-type particles were also observed in the intercellular spaces of tumour-free hepatic tissues, but no budding was seen in tumour-free areas. Large amounts of virus particles were seen also in the spleen both extracellularly and in intracellular vacuoles. No kidney tumours developed, but sporadic extracellular virus particles could be demonstrated in the kidneys. The primary hepatomas induced by MC29 virus in turkeys proved to be a very suitable model for the morphological study of interrelations between oncogenic viruses and eukaryotes.

GENETIC CONTROL OF RESPONSE TO ROUS SARCOMA VIRUSES
IN RHODE ISLAND RED FOWL

CS. DRÉN, P. K. PANI and L. N. PAYNE

*Veterinary Research Institute of the Hungarian Academy of Sciences, Budapest and Houghton
Poultry Research Station, Houghton, U.K.*

It was shown in a light breed White Leghorn fowl (WL) that the response to infection with Rous Sarcoma virus subgroups A and C was controlled by genes at the two linked loci, *tva* and *tvc*, respectively. In the present work, a heavy breed of Rhode Island Red (RIR) fowls was subjected to similar studies to investigate, (a) the single gene control of host response to viruses of these two subgroups; (b) the hypothesis that the *tva* and *tvc* loci are linked like in WL. Two RIR sire families, consisting of 8 dams per family, were chosen at random, and pedigreed cultured embryos were individually challenged with the two subgroup viruses. On the basis of the progeny response to the two viruses, putative genotypes of the parents were allocated. These parents were test-mated with WL parents of known genotype, $a^r a^r c^r c^r$, and the patterns of segregation of resistant and susceptible phenotypes were studied within families. Results have shown that the host response of RIR fowl to viruses of these two subgroups, A and C, is under the control of the *tva* and *tvc* loci, respectively, and that the two loci are linked as in WL fowl.

HETEROKARYONS BETWEEN CHICK ERYTHROCYTES AND SV₄₀
INDUCED H-50 TUMOUR CELLS

GY. SZÜCS and M. KELLERMAYER

Public Health Station, Pécs and Institute of Clinical Chemistry, University Medical School, Pécs

Efforts were made to produce heterokaryons between SV₄₀-induced H-50 hamster tumour cells and chick erythrocytes (CE). This system needs larger amounts of inactivated Sendai virus than the HeLa-CE and HEp-2-CE systems. A 70–75% fusional rate was found at 4000–8000 haemagglutinating units. The growth rate as well as the biological functions of the heterokaryons were followed by incorporation of labelled precursors. Incorporated [³H]thymidine in homologous and heterologous nuclei as well as the rate of thymidine incorporation depended on the time elapsed after fusion. It is concluded that the heterokaryons produced in these experiments are suitable for further analysis of biological activity of the nuclei and of the properties of the integrated virus genome.

CLINICAL TRIALS WITH INTERFERON INDUCING DOUBLE-STRANDED RNA

L. BORECKÝ, J. DOSKOČIL, V. LACKOVIČ, V. BUCHVALD, E. MALÝ,
Z. GRUNTOVÁ, E. OBRUČNIKOVÁ, M. OBRUČNIK, A. ŽUFFA and
M. HAVRÁNKOVÁ

Institute of Virology, Slovak Academy of Sciences, Bratislava, Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague, Dermatology Departments, University Hospitals, Bratislava and Košice, Department of Ophthalmology, University Hospital, Olomouc, Institute of Histology, University of Olomouc, Bioveta, Nitra and Slovak Oncological Institute, Bratislava, Czechoslovakia

During a 4 year period, more than 100 patients and a similar number of domestic animals with skin and eye diseases of viral aetiology as well as a small number of skin diseases of non-viral or unclear aetiology were treated with ointments containing double-stranded RNA (ds-RNA). The preparation of ds-RNA was extracted from impermissible *Escherichia coli* bacteria infected with phage f2, and purified by gel filtration and electrophoresis. No indication of absorption, toxicity or mutagenicity was obtained in toxicological tests. The results shown that ds-RNA can successfully be used in the therapy of viral skin and eye diseases.

IN VITRO "AGEING PROCESS" AND INTERFERON INDUCTION
IN MOUSE PERITONEAL CELLS

E. SZOLGAY and M. TÁLAS

Microbiological Research Group of the Hungarian Academy of Sciences, Budapest

Nucleic acid and protein synthesis, and the Newcastle Disease Virus-induced interferon production were investigated in mouse peritoneal cells cultured *in vitro*. Interferon production and [³H]thymidine incorporation decreased in aged cell cultures, while [³H]uridine and [¹⁴C]amino acid incorporation and the viability of cells showed little or no change. Cell-free culture fluids from the cultivated peritoneal cells inhibited both [³H]thymidine incorporation in, and interferon production by, freshly explanted peritoneal cells.

A SIMPLE BATCH ION EXCHANGE METHOD FOR DEMONSTRATION
OF SPECIFIC IgM ANTIBODIES

G. NAGY, I. MEZEY and E. MOLNÁR

National Institute of Hygiene, Budapest

A simple batch ion exchange method has been elaborated for the separation of IgM immunoglobulins. The method allows simultaneous fractionation of IgM antibodies from a number of sera within hours, using small volumes

and no special laboratory equipment. Comparing ion exchange column chromatography with the batch method for fractionation of IgM antibodies from 52 sera with specific antibodies to tick-borne encephalitis virus and from 10 sera with those to rubella virus, practically identical results were obtained. Nevertheless the basic dilution of IgM antibodies proved to be somewhat higher with the batch technique than with column chromatography. The procedure offers a possibility for routine virus laboratories engaged in virus serology for the demonstration of specific IgM antibodies.

USE IN THE DIAGNOSTICS OF TICK-BORNE ENCEPHALITIS OF A RAPID
MICROMETHOD BASED ON SPECIFIC IgM ANTIBODY DEMONSTRATION

G. NAGY, I. MEZEY and E. MOLNÁR

National Institute of Hygiene, Budapest

A hundred and seventy-nine serum samples from 95 patients with encephalitis or meningitis were tested for specific IgM antibodies after heparin-manganese-chloride treatment. Preliminary tests had shown that all samples contained antibodies to tickborne encephalitis (TE) virus. Separation by ion-exchange chromatography was carried out in 137 serum samples of 79 patients. IgM antibodies reacting with TE virus antigen were demonstrated in 101 samples (74%) of 66 patients (83%). Ion-exchange batch separation was performed in 94 samples from 51 patients, and 67 samples (71%) from 43 patient proved to be positive in the IgM haemagglutination-inhibition (HI) test. Of the 52 samples tested parallel with both methods, 48 gave identical results. Forty-six unfractionated serum samples from 84 patients were pretreated with 2-mercaptoethanol (2-ME), and in this way 41 samples (49%) from 32 patients (70%) showed a significant titre fall attributable to inactivation of IgM antibodies reacting with the TE virus. In samples taken in the first few weeks after onset, pretreatment with 2-ME and the IgM separation methods yielded comparable results. Paired caolin-absorbed serum samples from 95 patients were tested for TE antibodies without any further treatment. HI titre rise was demonstrable in 37 cases (39%) only, while 53 additional cases proved to be positive when the same sera were tested for specific IgM. It is recommended to supplement TE serodiagnostics with 2-ME pretreatment of early sera and ion-exchange batch IgM separation.

PATHOGENESIS OF SWINE FEVER DISEASE: REACTIONS OF PIGS
HAVING RECEIVED SUSPENSIONS OF PANCREAS OF NORMAL PIGS
AND CHYMOTRYPSIN

G. KORN and W. MATTHAEUS

Federal Institute of Virus Research, Tübingen, G.F.R.

Different kinds of antibodies have been described in swine fever disease. The neutralizing antibodies are directed against viral antigens. Those antibodies that precipitate in pancreas immunodiffusion test and those labelled with fluorisothiocyanate react with the cell pancreas-antigens. One of the latter is chymotrypsin(ogen) another one an enzyme with ATP-ase activity. Although antibodies are produced, there is evidence against a direct pathogenic effect of the virus. For that reason the question emerged of the pathogenicity of the cell-pancreas-antigens, especially of chymotrypsin(ogen). Pigs treated with suspension of pancreas of normal pigs and with isolated bovine pancreatic chymotrypsin developed a syndrome identical to that of swine fever disease. Chymotrypsin can be detected in the serum of pigs suffering from severe swine fever disease, and hypocoagulability too, is observed. In the case of virus replication in the cells of the lymphomyeloid complex, increased production and/or release of chymotrypsin(ogen) is assumed to occur. Activation of chymotrypsin then results in a disorder of the enzyme system. Thus, swine fever disease may be interpreted as a toxic disease with chymotrypsin(ogen) precipitating factor.

TICK-BORNE ARBOVIRUSES ISOLATED IN CZECHOSLOVAKIA

M. GRESIKOVÁ

Institute of Virology, Slovak Academy of Sciences, Bratislava, Czechoslovakia

Tick-borne arboviruses are distributed all over Europe; they are of importance mainly in the temperate zone. All arboviruses which are vectored by ticks and classified in antigenic group B (Flavivirus, Kemerovo and ungrouped) have been isolated in Czechoslovakia. For circulation of tick-borne arboviruses in nature the following conditions are of importance: abundance of vectors and maintenance hosts; climatic conditions which enable the virus to multiply in vectors and to survive the extrinsic incubation period; type of microhabitat and animal behaviour and coincidence of host population with seasonal activity of vectors and periodic fluctuation of the ecosystem. Human influence upon certain biogeocoenes also plays a role. Studies on the ecology of tick-borne encephalitis virus are presented in a film.

Bacterial genetics

TRANSFER OF DRUG-RESISTANCE PLASMIDS IN STAPHYLOCOCCI DURING MIXED INCUBATION OF DONOR AND RECIPIENT STRAINS

W. WITTE

Institute of Experimental Epidemiology, Wernigerode, G.D.R.

This kind of transfer was first described by LACEY in 1971. Transfer is not a transformation: treatment of the incubation mixture with DNase has no influence on the frequency of transfer of Pen and Chl resistance plasmids. Transduction can probably be also excluded: immunoglobulin G, which inhibits phage adsorption unspecifically, exerts no influence on the transfer frequency: it is also possible to transfer plasmids to encapsulated strains. The capsular material of staphylococci is known to inactivate phages. A prerequisite to transfer is probably a close contact of donor and recipient cells; dilution of the incubation mixture for transfer leads to an unproportional decrease of the transfer frequency. Transfer frequency is decreased by treatment with methicillin. It is possible to transfer chloramphenicol-resistance plasmids from *Staphylococcus epidermidis* to *Staphylococcus aureus* and *vice versa*.

PHENOTYPE OF METHICILLIN RESISTANCE IN STAPHYLOCOCCUS AUREUS

F. ROZGONYI, J. KISS, G. LÉVAL, P. BIACS and L. VÁCZI

Institute of Microbiology and Institute of Anatomy, Histology and Embryology, University Medical School, Debrecen and Institute of Agricultural Chemistry Technology, University of Technical Sciences, Budapest

Comparisons were made of a methicillin-resistant (MR) natural mutant with its methicillin-sensitive (MS) counterpart both derived from a naturally occurring MR isolate of heterogeneous population. MR mutant 5814R had a significantly longer generation time than the MS substrain 5814S. On the surface of the cells of the MR mutant a circumferential continuous and tortuous material was seen to join the outer layer of the wall without demarcation; it was assumed to be the outmost part of the wall. The circumferential material was absent from the surface of the MS cells. MR cells contained a significantly higher amount of lipids than their MS counterparts. The fatty acid composition of the individual phospholipids from the two substrains appeared to be remarkably different. At elevated temperature the phenotypic expression of methicillin resistance diminished in the MR mutant, whereas the genotype of methicillin resistance remained unaffected.

CHANGES IN PHAGE PATTERN IN STAPHYLOCOCCUS AUREUS
DUE TO ELIMINATION OF DIFFERENT PLASMIDS

É. BÁN and L. JÁNOSI

Central Hospital for Infectious Diseases, Budapest and National Institute of Hygiene, Budapest

Following plasmid elimination with different curing agents (acridine orange, ethidium bromide, sodium dodecyl sulphate, rifampicin, heat) the phage pattern changed only in one strain, where the loss of penicillin and cadmium resistance markers resulted in sensitivity to phages 79, 85, 95, AC₁, 88 and 90 whereas lysogenicity remained unchanged. Transduction experiments showed that acquisition of plasmid carrying penicillin and cadmium resistance markers resulted in a restricted phage pattern.

R FACTORS OF ESCHERICHIA COLI AND SALMONELLA TYPHI-MURIUM

H. MILCH

National Institute of Hygiene, Budapest

Phage-restriction (P.R.) and phage-modification (P.M.) activity of R factor and the occurrence of other transferable plasmids in antibiotic resistant *Escherichia coli* and *Salmonella typhi-murium* strains of different origin were investigated. (i) Out of 158 human pathogenic *E. coli* strains 92 carried R factors, 56 of which exhibited P.R. or P.M. activity. Out of 31 R factors demonstrated in the 53 pig-pathogenic *E. coli* strains tested, 23 yielded P.R. or P.M. Out of 21 calf-pathogenic *E. coli* strains 11 carried R factors, 3 of which had P.R. or P.M. activity. Out of the 6 *E. coli* strains recovered from chicken, two contained R factor with P.R. and P.M. activity. (ii) Out of 85 antibiotic resistant *S. typhi-murium* strains 24 carried R factor, 10 of which had P.R. activity. (iii) The widest restriction-patterns were displayed by the R factors of the pig-pathogenic *E. coli* strains. (iv) In pig pathogenic *E. coli* strains Col and Hly plasmids were also present. The P.R. and P.M. effects of Hly plasmid have been demonstrated. (v) Phages restricted by *Salmonella* and *Escherichia* R factors were identical, only the restriction patterns differed. (vi) Changes of *S. typhi-murium* phage type 1 were caused by R factors derived from both *E. coli* and *S. typhi-murium* strains.

R FACTORS IN *SALMONELLA* TYPHI STRAINS

V. G. LÁSZLÓ

National Institute of Hygiene, Budapest

Antibiotic sensitivity of 2227 *Salmonella typhi* strains was tested. Three strains isolated from different carriers were resistant to (1) streptomycin, chloramphenicol, tetracycline, ampicillin, sulphonamides; (2) chloramphenicol, tetracycline, ampicillin; and (3) tetracycline. The resistance was due to R factors. Change in phage type was associated with one of the R factors: type E_{1a} changed to untypable. The same R factor was shown in an *Escherichia coli* strain isolated from the faecal flora of the corresponding carrier. All of the R factors were *fi*⁻ and two of them belonged to compatibility group H1. *E. coli* phages were restricted by two R factors, *S. typhi* phages by one.

PLASMID CURING MECHANISM OF CHLORPROMAZINE

Y. MÁNDI, J. MOLNÁR and I. B. HOLLAND

Institute of Microbiology, University Medical School, Szeged and Department of Genetics, University of Leicester, Leicester, England

In a previous study chlorpromazine (CPZ) caused efficient curing of an R-factor from *Escherichia coli* strain RC 709 Sm^rT^rSu^rCl^r and of the F-prime plasmid from *E. coli* K12 LE 140. Recently all the R⁻ bacteria cured by CPZ were found to be tetracycline sensitive. Only 90% of them had, however, lost the Su, Sm, Cl resistance determinants together, and 10% of the tetracycline sensitive bacteria remained resistant to one or another of the mentioned antibiotics. After growth in the presence of 60 µg/ml CPZ, 70% of *E. coli* K12 LE 140 became lac⁻. The MS-2 pilus specific phage sensitivity of the cured bacteria, and that of the plasmid-carrying bacteria was the same. It was assumed that genes of R-factor, or of F-prime plasmid could be eliminated separately. If *E. coli* K12 LE 140 was grown in the presence of CPZ at a concentration 40–60 µg/ml, 5–50% of the colonies developed mucoid surface on agar media. Bacteria picked up from the mucoid colonies were similar to the host strain, indicating that their appearance was not due to a selective mechanism.

ANTIBACTERIAL AND PLASMID-CURING EFFECT OF SOME TRICYCLIC COMPOUNDS ON *ESCHERICHIA COLI* K12 F⁺lac

J. MOLNÁR, Y. MÁNDI, I. B. HOLLAND and G. SCHNEIDER

Institute of Microbiology, University Medical School, Szeged, Department of Genetics, University of Leicester, Leicester, England and Institute of Organic Chemistry, József Attila University of Sciences, Szeged

The antibacterial and plasmid curing effect of different tricyclic compounds on *Escherichia coli* strain K12 LE 140 was studied. The minimum inhibitory concentrations were, amitriptyline, 160; imipramine, 180; diethazine, 160; phenothiazine, 500; tilorone, 600; and rivanol 20 µg/ml, in MTY media. Chlorpromazine sulphoxide, tetraoxyanthraquinone and fluorescein had no antibacterial effect even at a concentration of 1000 µg/ml. The following antibacterial compounds partly eliminated the F⁺lac-plasmid: amitriptyline (150 µg/ml) 70%, imipramine (160 µg/ml) 40%, diethazine (40 µg/ml) 22%, phenothiazine (400 µg/ml) 0.1%, tilorone (400 µg/ml) and rivanol (10 µg/ml) 1%. Fluorescein, tetraoxyanthraquinone and chlorpromazine sulphoxide were ineffective as plasmid curing agents even at a concentration of 1000 µg/ml. The plasmid-curing effect of imipramine was very low in the presence of fluorescein or tetraoxyanthraquinone. The plasmid-eliminating action of imipramine was the most effective at 37 °C under semianaerobic conditions. The electronic state of tricyclic molecules may have an important role in their antibacterial and plasmid-curing effect.

A RESIDENT PLASMID IN *RHIZOBIUM MELILOTI*

J. HORVÁTH, I. BARABÁS and T. SIK

Institute of Genetics, Biological Research Centre of the Hungarian Academy of Sciences, Szeged

Wild type, non-lysogenic *Rhizobium meliloti* was treated with a plasmid elimination method worked out for induced lysogenic bacteria to eliminate phages as episomes. The aim was to isolate a *Rhizobium* free of its natural resident plasmid, and to identify the genetic information carried by the plasmid. Mutants were isolated by a single treatment with acriflavine at high concentration or successive growth with acriflavine at low concentration. Changes in phage sensitivity, nitrate-reductase activity and antibiotic resistance were followed in vegetative mutants and nodulation. Changes in effectiveness (nitrogenase activity) were studied in symbiosis. The isolation of *Rhizobium* plasmid DNA and its physico-chemical characterization was carried out.

PHENOTYPIC REVERSION OF NITROGENASE BY PURINE BASES
IN A PLEIOTROPHIC MUTANT OF RHIZOBIUM MELILOTI

I. BARABÁS and T. SIK

Institute of Genetics, Biological Research Centre of the Hungarian Academy of Sciences, Szeged

The *Rhizobium meliloti* pleiotropic mutants isolated were defective both in nitrate-reduction by vegetative forms and in nitrogen fixation in the symbiotic association with lucerne. One of these mutants studied in detail was not auxotrophic. It was able to form nodules on the host plant, but exhibited no nitrogenase activity as measured by acetylene reduction. The nitrogenase activity could be restored in 80–100% by adding 10–50 µg/ml of adenine, guanine or hypoxanthine to the symbiotic system at the time of inoculation of the host plant with the mutant. Neither the growth of vegetative bacteria was stimulated nor the nitrate-reductase activity was restored by purine bases at 1–100 µg/ml.

AMMONIA ASSIMILATION IN RHIZOBIUM MELILOTI

Á. KONDOROSI, Z. SVÁB, G. B. KISS and R. A. DIXON

*Institute of Genetics, Biological Research Centre of the Hungarian Academy of Sciences, Szeged
and ARC Unit of Nitrogen Fixation, University of Sussex, Brighton, England*

Enzymes involved in ammonia assimilation of *Rhizobium meliloti* were studied. High levels of glutamine synthetase and glutamate synthase were found in cells grown in media containing ammonia at either low or high concentration. Alanine dehydrogenase was present only in low amounts. Glutamate dehydrogenase could not be detected. Mutants lacking glutamate synthase enzyme activity were isolated and characterized. These mutants could grow only in glutamate-supplemented medium and were fully effective in symbiotic nitrogen fixation. The revertants showed the characteristics of the wild type strain. These findings indicate that ammonia is assimilated via the glutamine synthetase and glutamate synthase pathway in this strain. The function of glutamate synthase is not required in symbiosis. It is suggested that the ammonia produced in symbiotic nitrogen fixation is then assimilated by enzymes from the plant.

ISOLATION AND CHARACTERIZATION OF ASSIMILATORY
NITRATE-REDUCTASE MUTANTS FROM RHIZOBIUM MELILOTI

G. B. KISS, Á. KONDOROSI and Z. SVÁB

Institute of Genetics, Biological Research Centre of the Hungarian Academy of Sciences, Szeged

The functional role of assimilatory nitrate-reductase in symbiotic nitrogen-fixation was investigated in *Rhizobium meliloti*. A new and direct selection technique was worked out for the isolation of mutants unable to utilize nitrate as sole nitrogen source. Mutants were characterized biochemically and tested for effectiveness in symbiotic nitrogen fixation.

GLUTAMINE SYNTHETASE AND NITROGEN FIXATION
IN RHIZOBIUM MELILOTI

Z. SVÁB, Á. KONDOROSI, K. BARNA, G. KISS and R. A. DIXON

Institute of Genetics, Biological Research Centre of the Hungarian Academy of Sciences, Szeged and ARC Unit of Nitrogen Fixation, University of Sussex, Brighton, England

In free-living, nitrogen-fixing organisms synthesis of nitrogenase is positively regulated by the enzyme glutamine synthetase. We studied the relationship of glutamine synthetase and nitrogenase in symbiotic nitrogen-fixing bacteria. A mutant, requiring glutamine for optimum growth was isolated and further characterized. It exhibited a low glutamine synthetase activity as compared to the wild type bacterium and did not fix nitrogen in symbiosis. It is suggested that glutamine synthetase is involved in nitrogen fixation of symbiotic nitrogen-fixing bacteria as well.

LETHAL EFFECT OF NITROUS ACID ON ESCHERICHIA COLI

ZS. NAGY, M. SOLYMOSSY and F. ANTONI

First Institute of Chemistry and Biochemistry, Semmelweis University Medical School, Budapest

To show the primary site of mutagenic action of nitrous acid, stationary phase *Escherichia coli* strains of different DNA repair capacity ($uvrA^-$: $recA^-$: $polA^-$, and wild type) were examined. The cell membrane transport function of the cells was damaged by 3 min incubation with 0.25 M HNO_2 ; as measured by $[^{14}C]$ leucine and $[^3H]$ glucose uptake, after 15 min a 72% inhibition of leucine transport occurred. Inhibition of DNA synthesis and breaking up of the DNA chain, which was but slightly influenced by the repair capacity of

the bacteria, appeared after 20 min exposure. The decrease in the number of viable cells showed a correlation with the degree of transport function inhibition. The finding suggested the possibility of a "non-DNA target", in the lethal effect of HNO_2 .

MAPPING FURTHER PORPHYRIN LOCI IN *BACILLUS SUBTILIS*

A. MICZÁK and GY. IVÁNOVICS

Institute of Microbiology, University Medical School, Szeged

Three genes (*hemE*, *hemF*, *hemG*) taking part in the porphyrin biosynthesis of *Bacillus subtilis* were mapped by two and three-factor transduction crosses. The gene *hemE* determines uroporphyrinogen decarboxylase (EC 4.1.1.37), the gene *hemF* coproporphyrinogen oxidase (EC 1.3.3.3) and the gene *hemG*, ferrochelatase (EC 4.99.1.1). The loci *hemE*, *hemF*, *hemG* are not linked to the *hemA* locus and are located near the *argC* and *metD* loci. These *hem* genes replicate simultaneously with the first three porphyrin genes, which are situated on the other replication arm (between *leu-1* and *pheA* loci) of the chromosome. Mutants bearing the *hemA*, *hemB*, *hemC* markers grow in the presence of haemin and cysteine, while the others need no cysteine. It is assumed that in the case of the first three mutants besides porphyrin synthesis the synthesis of corrinoids is also absent. This synthesis starts from porphobilinogen and only *hemE*, *hemF*, *hemG* mutants are able to synthesize and convert this compound. Further investigations are necessary to determine the role of cysteine.

ECOLOGY OF TEMPERATE PHAGES HARBOURED BY *BACILLUS CEREUS*

E. NAGY, B. PRÁGAI and GY. IVÁNOVICS

Institute of Microbiology, University Medical School, Szeged

An RNA phage, known as AP50, containing phospholipids was isolated from soil some years ago. This temperate phage was highly specific for some strains of *Bacillus anthracis* and was similar to phage Wbeta. It was assumed to derive from a lysogenic strain of some *Bacillus* species. A number of *Bacillus cereus* strains were isolated from soil and tested for lysogeny. Most of the strains were double lysogenic. The W strain of *B. cereus*, beside the beta prophage, carried another prophage termed *wx*, responsible for phospholipase A (megacin A) production upon induction of the strain. Five *B. cereus* strains producing bacteriocin carried an anthrax specific phage very similar to AP50, besides

a *wx* type prophage. These anthrax specific phages of the mitomycin C induced (1 $\mu\text{g/ml}$) concentrated lysate of the *B. cereus* strains were identical in morphology, host range and chemical structure with phage AP50. The *wx*-like and AP50-like phages with their interesting chemical and biological characteristics form new species *Bacillus* phages.

PHAGE-DEPENDENT CHANGES IN SHIGELLA FLEXNERI TYPE ANTIGEN SYNTHESIS

I. FINANCSEK, I. KÉTYI, W. SASAK, W. JANKOWSKI, E. JANCZURA and
T. CHOJNACKI

*Institute of Microbiology, University Medical School, Pécs and Institute of Biochemistry and
Biophysics, Polish Academy of Sciences, Warsaw, Poland*

In lysogenic conversion, the origin of the information is a basic question. The information may be carried by the phage-genome, or by a prophage-derepressed bacterial gene. The following findings support the assumption that, in lysogenized *Shigella flexneri*, information for type antigens is coded in the phage genome. (i) Some mutant phages isolated were incapable of causing complete antigenic conversion or induced only a modification of the antigenic structure. (ii) Some phage mutants of thermo-sensitive antigen-converting ability were isolated. Incorporation of glucose, a chemical component of the type antigen, was allowed at a permissive temperature (30 °C), but inhibited at a non-permissive (42 °C) one. (iii) Some phage mutants with missing converting ability were able to provoke antigenic conversion in a *S. flexneri* strain carrying a suppressor gene.

LYSOGENIC CONVERSION OF MYCOBACTERIUM SMEGMATIS STRAIN RABINOWITZ

K. HÁBER and B. VAJDA

Microbiological Research Group of the Hungarian Academy of Sciences, Budapest

Cells of *Mycobacterium smegmatis* strain Rabinowitz were lysogenized by V72 (Rabinowitz) phages. Lysogenized cells of the Rabinowitz strain, in contrast to the original strain, proved sensitive to the phage of *M. smegmatis* strain *butyricum*. The *butyricum* phages were able to adsorb to the cells of strain Rabinowitz as well; however, they formed plaques on it only at a frequency of 10^{-8} , while the frequency for the lysogenic cells was 5×10^{-1} . The burst size of phage *butyricum*-producing *M. smegmatis* strain *butyricum* cells was 60 and that of lysogenic *M. smegmatis* strain Rabinowitz cells only 6.

UV-REPAIR IN MYCOBACTERIUM SMEGMATIS STRAIN RABINOWITZ

B. VAJDA and K. HÁBER

Microbiological Research Group of the Hungarian Academy of Sciences, Budapest

Cells of *Mycobacterium smegmatis* strain Rabinowitz were damaged by varying doses of ultraviolet light, then survival of the cells was analysed under different conditions. Illuminating the UV-damaged cells by visible light increased the survival 50-fold compared to cells incubated in dark. Maximum photo-reactivation was achieved if the UV damaged cells were illuminated for 150 min. The increase of survival of UV-damaged cells was only 3-fold after repair in dark and this could not be inhibited by caffeine. In other experiments phage V72 of strain Rabinowitz was damaged by UV-light and the dose response (number of plaque-forming units) was determined. The *M. smegmatis* strain Rabinowitz cells were able to photoreactivate also the UV-damaged V72 phages.

THE METHYL-BASES OF DNA OF MYCOBACTERIA AND MYCOBACTERIO-PHAGES

P. SOMOGYI and I. FÖLDES

Microbiological Research Group of the Hungarian Academy of Sciences, Budapest

Methyl-bases of the DNA of two mycobacterial species (*Mycobacterium smegmatis* strain *butyricum* and *Mycobacterium phlei*) and their phages were studied. Cultivation of mycobacteria and phage production were performed in the presence of methionine labelled with ^3H at the methyl group. After isolation of DNA and hydrolysis with 72% perchloric acid or 1 N hydrochloric acid the labelled bases were studied with thin layer chromatography. (i) Marked differences could be demonstrated between the DNA-base-ratios of the bacteria and their phages. (ii) The DNA of *M. smegmatis* strain *butyricum* and its phages contained 5–6 times more labelled bases than the DNA of *M. phlei* and its phages. (iii) Chromatography showed that (a) although the DNA-base-ratios of *M. smegmatis* strain *butyricum* and its phages were different, they contained two labelled bases with the same R_F values after hydrolysis with 72% perchloric acid. In chromatograms obtained after hydrolysis with 1 N hydrochloric acid, the same labelled bases could again be demonstrated showing that both corresponded to purine bases. (b) Methyl-bases of the DNA-s of *M. phlei* and its phages could be most likely identified as 5-methyl-cytosine. The role of methyl bases in the synthesis of DNA of mycobacteria and mycobacteriophages will now be investigated on the basis of the described studies.

DELETION AND THE THREE POINT MAP OF GENE C OF
RHIZOBIOPHAGE 16-3

L. OROSZ and Z. ASCHER

Institute of Genetics, Attila József University, Szeged

(i) Usual criteria (reversion, recombination pattern) have shown that out of 75 spontaneous clear mutants 16 were deletion ones. (ii) Mutant del CJZ 5 covers all the known mutations and exhibits a pleiotropic effect on phage growth. (iii) Deletions with one end outside and the other inside the gene C (e.g. del CJZ 31) always penetrates from the "silent" left side and never from the right "gap" defined by gene C and the nearest essential early mutation ts 5124. (iv) Certain deletions, irrespective of size, recurred 2-8 times. (v) No contradiction was found between deletions and the preestablished map order constructed by intracistronic 3 point crosses. (Two point crosses with the same set of mutants have shown non-additivity and marker effect). (vi) The latter observation could be explained in terms of current crossing-over models (HOTCHKISS; MESELSON and RADDING; SZYBALSKI) if minimum limits for the length of heteroduplex and gene conversion are introduced.

TWO POINT CROSSES WITH INTERNAL RECOMBINATION CONTROL
TO DETECT MARKER EFFECT IN GENE C OF RHIZOBIOPHAGE 16-3

K. ROSTÁS and L. OROSZ

Institute of Genetics, Attila József University, Szeged

From ti4 conditional clear (heat inducible) mutant of temperate phage 16-3, non-conditional double mutants (ti4-C) were isolated. After reversion tests, mutants producing only ti4 "turbid" revertants were selected. This pattern is consistent with ti4-C, whereas C alone would lead to a non-conditional mutant phenotype. Double mutants of this type are useful in fine mapping. The aim of the study was to observe the specific effect of C mutations and to estimate anomalies in two point mapping. In order to standardize the two point crosses ts mutation in the late genes ts4 and ts6 were introduced separately into each clear mutant. Recombination between the closely linked ts marker ($R 10^{-3}$) served as internal control for recombination between C mutations — i.e., the No. of ti4 recombinants observed at 28 °C were normalized to the No. of ts⁺ recombinants selectively plated at 36 °C. No. ti4 per No. ts⁺ ratio in a pair of crosses — i.e., ti4-Cx-ts4 × ti4-Cy-ts4 and ti4-Cx-ts6 × ti4-Cy-ts6 defines normalized R between Cx and Cy. All the possible pairs of crosses were carried out with the mutants ti4-C (sp2; sp3; sp4; U6;

U7; U8) ts4 and ts6 sets as well as with K-ts4 and K-ts6. It has been shown that (i) normalized R varies from 0.5 up to 10; (ii) the order of sp4-sp3-U8-U6 is apparently established, in contrast to K; U7; sp2; (iii) a tendency of additivity is consistent with more than half of the recombination data. Two point map order is more or less colinear with three point and deletion maps. (iv) The marker effect of mutation U8 reduced R by a factor of two and crosses, where one of the parents was K, have shown apparent non-additivity with mutants right to it (U7; U8; U6).

MICROBIOLOGICAL ANALYSIS OF A NEW RESTRICTION SYSTEM

M. SZEKERES

Institute of Genetics, Attila József University, Szeged

KISS *et al.* (1976) purified and characterized a new restriction endonuclease Endo R Bsp R from *Bacillus sphaericus* R, with some advantageous properties. In the present experiments this restriction system was analysed microbiologically. A cross infection system between R, non-restricting C 23 and C 25, was constructed by the use of phage 1025 A. The restriction pattern of *B. sphaericus* R was investigated. Values of relative efficiency of plating concerning the non-restricting strains were as follows. Phage 1025 A grown on R: 0.1 (R) and 1 (C 23 and C 25); phage 1025 A grown on C 23 and C 25: $2-3 \times 10^{-3}$ (R) and 1 (C 23 and C 25). *Bacillus subtilis* X 5, infectable for phage 1025 A, has a well characterized restriction system possessing the same restriction site: 5' . . . GGCC . . . 3'. E.o.p. data have shown that phage 1025 A propagated in *B. subtilis* X 5 cells was protected against Endo R Bsp R action, suggesting an identical position of the methylated cytosine in the recognition sequence.

ADSORPTION OF SP50 PHAGE ON COMPETENT CELLS OF BACILLUS SUBTILIS

J. FÖLDES, É. VARGA and K. DOMONKOS

Institute of Microbiology, University Medical School, Szeged

To analyse the mechanism of transfection, a method has been elaborated which eliminates the possibility of phage infection. The background of the dissociation of the two processes was examined by adsorbing SP50 phage on competent and incompetent cells of *Bacillus subtilis* 168 indole auxotroph in synthetic and complete medium. The adsorption constant was calculated by

determining the number of adsorbed and free phages according to ADAMS's method. (i) Phage receptors were present in both competent and incompetent cells. (ii) The adsorption rate of SP50 phage was identical on both kinds of cell and was very slow in synthetic medium ($K = 8 \times 10^{-10}$ ml/min) and more rapid in peptone-yeast extract medium ($K = 10^{-9}$ ml/min).

BIOCHEMICAL AND GENETIC STUDIES ON THE COMPETENCY OF *BACILLUS SUBTILIS* 168

J. FÖLDES, K. DOMONKOS and É. VARGA

Institute of Microbiology, University Medical School, Szeged

The uptake and fate of foreign nucleic acid in *Bacillus subtilis* were examined by studying (i) the nuclease and heat sensitivity (melting point) of transfecting and transforming nucleic acids; (ii) nuclease activity of the host and (iii) association between competency and certain genetic markers. (i) In a given population of *B. subtilis* 168 competent cells, transforming DNA is taken up more rapidly than transfecting DNA; the time of nuclease sensitivity is 5–8 and 30–45 min, respectively. (ii) Denaturation of transforming DNA occurs at 100 °C after 10 min, that of transfecting DNA at 90 °C within 10 min. (iii) Extracts with nuclease activity prepared from prototrophic and from competent and incompetent auxotrophic bacteria, depending on the physiological and genetic characteristics of the cells, decrease or destroy the transforming and transfecting effects of nucleic acids. (iv) The prototrophic (wild-type) strains cannot be transfected, whereas the different isogenic auxotrophs are liable to transfection. (v) Auxotrophic strains transformed into prototrophic cells with the DNA of the wild type culture retain their transfectability. It is concluded that (i) the uptake and fate of foreign nucleic acid depend, in addition to the structural properties of DNA, on the physiological state of the cells characterized by competency and nuclease activity; (ii) the metabolic mutations examined and transfectability are not linked genetically.

ROLE OF CELL WALL AND MEMBRANE OF TRANSFECTED *BACILLUS* *SUBTILIS* IN THE BIOSYNTHESIS OF SP50 PHAGE

J. FÖLDES, É. VARGA and K. DOMONKOS

Institute of Microbiology, University Medical School, Szeged

To confirm that phage biosynthesis occurs on the cell membrane of the host, samples were taken at intervals after transfection, the cell wall of the bacteria was removed and different sucrose gradient fractions of the proto-

trophs were counted for plaque-forming units. It has been shown that (i) after removal of the cell wall, about half of the p.f.u. remains in the membrane fraction; (ii) initiation of phage-DNA replication terminates after removal of the cell wall; (iii) the progressing replication of phage-DNA is completed in the protoplasts but on further incubation there is no increase in p.f.u.; (iv) in virion-infected and then protoplasted cells, phage synthesis continues according to the classical scheme. It is concluded that in transfected *B. subtilis* cells the cell wall and its enzymes play an important role in the initiation of phage-DNA replication. For phage synthesis in infected cells and in their protoplasts, the cell wall and the enzymes localized in it are not necessary.

MORPHOLOGICAL STUDIES ON VIRUS SYNTHESIS IN SP50 DNA TRANSFECTED BACILLUS SUBTILIS

J. FÖLDES, K. DOMONKOS, É. VARGA and É. SZABÓ

Institute of Microbiology and Department of Dermatology, University Medical School, Szeged

The finding that virus synthesis in *Bacillus subtilis* transfected with purified SP50 phage-DNA takes place on the cell membrane was checked by immuno-fluorescence technique using fluorescein-labelled goat anti-rabbit globulin. Samples taken between 0 and 150 min after transfection of *B. subtilis* were examined for the presence of phage particles. The highest number of plaque-forming units was shown at 150 min, whereas free (filtrable) phages began to appear at 150 min. Phages produced during transfection were demonstrable on the cell surface from the 60th min onward.

Bacteriology

THROMBOCYTOLYTIC ACTIVITY OF BACTERIAL PEPTIDOGLYCANS

M. RYC

Institute of Hygiene and Epidemiology, Prague, Czechoslovakia

Peptidoglycans of the bacterial cell wall exhibit some biological activities *in vivo* and *in vitro*. One of them — the thrombocytolytic activity — has been studied on the ultrastructural level. It has been postulated that peptidoglycans prepared by hot formamide or trichloroacetic acid extraction from the cell wall of group A streptococci are able to lyse blood platelets of different animal species, especially those of rabbits and rats. By this reaction 5-hydroxytryptamine is released. The efficient dose of peptidoglycan is low: initial changes in the ultrastructure of rabbit platelets occur after 10 min incubation

with 0.1 μg of peptidoglycan per ml. Rat platelets are less sensitive. The reaction requires the presence of complement and further plasmatic factors and in its development immunological mechanisms are involved. Peptidoglycans of other bacterial species (*Streptococcus pneumoniae*, *Staphylococcus aureus* and *Spirillum serpens*) exhibit a comparable effect. Therefore, this biological activity, similarly to some other endotoxin-like activities of peptidoglycan, seems to be a general phenomenon despite the previous opinion that bacterial peptidoglycans are structures which have only a mechanical function in the bacterial cell wall. For clarifying which part of the peptidoglycan is responsible for the described effect, different synthetic peptide and glycopeptide analogues were studied for their thrombocytolytic activity. It has been shown that peptides do not initiate the lysis of platelets but some synthetic glycopeptides are able to do so.

ANTIGENIC RELATIONSHIP AMONG FIMBRIAE OF ENTEROBACTERIACEAE

M. MULCZYK and M. NOWOTARSKA

*Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław
Poland*

The existence of a serological relationship of fimbriae among serotypes of the same genus and among genera of *Enterobacteriaceae* was determined by agglutination and absorption procedures. Fimbriae of different serotypes of the same genus, and among the genera: (a) *Escherichia*, *Shigella* and *Klebsiella*, and (b) *Salmonella*, *Arizona* and *Citrobacter* were related antigenically. No relationship was found among fimbriae of the genera: *Edwardsiella*, *Enterobacter*, *Hafnia*, *Serratia*, *Proteus* and *Providencia*. On the basis of the results obtained, nine antigenically distinct types of fimbriae were distinguished.

RAPID MICROBIOLOGICAL DIAGNOSIS OF CLOSTRIDIUM PERFRINGENS INFECTIONS

H. P. SCHAU

Institute of Hygiene, Erfurt, G.D.R.

A method is reported which allows the identification of *Clostridium perfringens* from clinical specimens in a few hours. Using a medium yielding optimum growth and toxin production, pure cultures are centrifuged and subjected to rapid tests (splitting of lactose, nitrate reduction, phosphatase and motility). The rapid method and the conventional technique gave the same results with 50 *C. perfringens* strains isolated from clinical samples.

GROUP AND TYPE DISTRIBUTION OF HAEMOLYTIC STREPTOCOCCI
IN SOMALI DURING THE YEARS 1975-76

J. SZITA, E. MÉRŐ, Zs. RÁCZ and I. SZABÓ

National Institute of Hygiene, Budapest

Between December 1975 and May 1976, 169 cultures, isolated in Mogadishu, Somali, were examined in the Streptococcus Laboratory of this Institute. The work was undertaken to compare the peculiarity of European and African streptococcal infections and strains. Out of 148 Somalian beta-haemolytic streptococci, 47.9% belonged to group A (in Hungary the corresponding incidence is 73.4%); the rest was distributed among groups B (17.6%), C (16.2%), G (15.5%), D (2.1%) and E (0.7%). *Streptococcus pyogenes* (group A) strains fell into 11 serotypes; these, except type 54, all occur in Hungary. Most of the isolates originated from skin infections. All complexes of PARKER's "impetigo" strains were met with (3,13,B₃₂₆₄; 8,25,Imp₁₉; 5,11,12,27,44) as well as other pyoderma strains: 14,49; 4,24; type 12 was frequent. Among other Lancefield groups the association of group B with urogenital infections was notable. Group A strains had an outstanding resistance against tetracycline (45.0%). The great number of skin infections may be explained by local circumstances.

INCIDENCE OF PSEUDOMONAS STUTZERI IN PATIENT WITH PULMONARY
DISEASES

Cs. CSUKÁS and P. JUHÁSZ

County Institute for Pulmonology, Szolnok

From the spring of 1974 onward, 27 *Pseudomonas stutzeri* strains were isolated from 22 patients. In 14 out of 27 specimens (18 sputum, 2 thoracic cavity, 6 urine and 1 bile samples) the organism was predominant, but never occurred in pure culture. Most strains from respiratory organs were obtained from patients with pneumonia; others originated from tumour, sarcoidosis, polycystic lungs and bronchitis. All strains were sensitive to kanamycin, gentamicin and polymyxin-B and most of them to tetracyclines, neomycin and colistin.

A SEROLOGIC STUDY OF ACINETOBACTER CALCOACETICUS STRAINS

M. M. ÁDÁM

National Institute of Hygiene, Budapest

Antigenic properties of 225 *Acinetobacter calcoaceticus* strains isolated from different specimens (sputum, respiratory tract, bile, urine, wounds hospital equipment) were studied to establish a typing schema including O and K antigens. Using passive haemagglutination test with the supernatant of bacteria boiled at 100 °C for 1 hr, except three untypable strains, the cultures were classified into 23 serogroups. K antigens were examined by slide agglutination, and by tube and microagglutination technique with living and formalinized cultures, respectively. Two of the 20 K antigen types had a complex structure. In 13 strains the envelope was not determinable because of auto- or polyagglutinability.

BRONCHOPULMONARY MYCOBACTERIOSES ASSOCIATED WITH MYCOBACTERIUM KANSASII

F. GIMPL and E. ZOLNAY

Institute of Pulmonology, Semmelweis University Medical School, Budapest

Out of 71 578 specimens cultured in the years 1975–1975 for mycobacteria, 3569 were positive for *Mycobacterium tuberculosis*: 13 samples yielded *Mycobacterium kansasii*, which is regarded as a facultatively pathogenic agent, mainly responsible for bronchopulmonary symptoms associated with atypical mycobacteria. Characteristics utilized in the identification of the species and criteria for the diagnosis of bronchopulmonary mycobacterioses are discussed.

TWO OUTBREAKS ASSOCIATED WITH MYCOPLASMA PNEUMONIAE

GY. MOLNÁR, J. SZITA, L. STIPKOVITS and M. TASS

National Institute of Hygiene, Budapest and Veterinary Research Institute of the Hungarian Academy of Sciences, Budapest

In September and November, 1975, two outbreaks of acute respiratory disease occurred in two counties involving more than 1000 patients weakly. Pneumonia was diagnosed in about half of the cases. One-hundred and fifty samples (swabs and sera) from 60 patients and 30 healthy persons were examined. From 26 patients mycoplasmas were cultured or the diagnosis was

confirmed by serological tests (21 *Mycoplasma pneumoniae*, 3 *M. hominis*, 2 *M. orale*). Examination of patients was repeated one month later. Three *Mycoplasma* strains were cultured from 30 swabs; the serum antibodies remained unaltered except in two sera showing increased titres.

SHEEP AS A SOURCE OF HUMAN MYCOPLASMA PNEUMONIAE INFECTION

L. STIPKOVITS, É. BENYEDA and J. MASSZI

Veterinary Research Institute of the Hungarian Academy of Sciences, Budapest and Municipal Hospital, Ajka

Mycoplasma pneumoniae was isolated from the lungs of serologically positive sheep handled by a 48-year-old man suffering from exudative erythema multiforme. He, his wife and his son were culturally and serologically proved to be infected with *M. pneumoniae*. In spite of erythromycin therapy, the man relapsed four times during one year. After treatment of the whole family with erythromycin and separation from the infected sheep, the skin lesions did not appear again during 3 years of observation. Artificial infection of serologically negative sheep with *M. pneumoniae* induced no specific clinical symptoms, but in the lungs of animals slaughtered 3 months after infection focal lesions of catarrhal pneumonia were present from which *M. pneumoniae* was recovered.

CHARACTERISTIC PROPERTIES OF CORYNEBACTERIUM PYOGENES VAR. HOMINIS

K. CSISZÁR

Public Health Station, Salgótarján

Three *Corynebacterium pyogenes* var. *hominis* strains isolated from the throat swabs of two patients and from crural ulcers were compared with *C. pyogenes* strains cultured from animals. All strains agglutinated and later haemolysed sheep erythrocytes. Immune serum prepared from one of the human strains agglutinated the other human as well as animal strains. In contrast, only human strains gave the haemolysin-inhibition test of ZÁHOROVÁ and KUBELKA and failed to attack gelatin.

SEROTYPING OF *ERYSIPELOTHRIX RHUSIOPATHIAE*

G. KUCSERA

State Institute for the Control of Veterinary Serobacteriological Products, Budapest

Within the species *Erysipelothrix rhusiopathiae* 16 serotypes and several subtypes have been distinguished. The double agar-gel diffusion test using autoclaved antigen proved to be the most suitable for serotyping. Erysipelas bacteria belonging to various serotypes differ in infectivity in different animal species. Numerous investigations have shown that a certain clinical picture of swine erysipelas is caused by a well-defined serotype of erysipelas bacteria. In other animals erysipelas-induced diseases and carriership are also restricted to definite serotypes. Serotyping of the isolates may, in some cases, be efficiently used for tracing the origin of infection.

TRANSMISSIBLE DRUG RESISTANCE OF *SALMONELLA* IN EAST SLOVAKIA

M. TARABČÁK, V. KRČMÉRY, J. JANOUSKOVÁ, M. HOCMANOVÁ
and M. BILČIKOVÁ

Regional Institute of Hygiene, Kosice, Research Institute of Hygiene, Bratislava and District Public Health Laboratory, Michalovce, Czechoslovakia

During the period 1952–1975, more than 60 000 human *Salmonella* strains belonging to 69 serotypes were isolated in East Slovakia. *S. typhi-murium* was a predominant serotype in each but 2 years, followed by *S. enteritidis*, *S. bareilly* and *S. anatum*. Since 1970, *S. kapemba*, *S. panama* and *S. agona* appeared among the most frequent serotypes. Until 1973, almost all strains of *Salmonella* were susceptible to antibiotics, although, since 1970, a sporadic incidence of *S. enteritidis*, *S. agona* and *S. typhi-murium* strains resistant to tetracycline and/or sulphonamides and/or streptomycin and/or ampicillin was observed. In 1973, in one district, *S. typhi-murium* strains resistant to at least 7 antibacterial drugs were encountered. Multiple drug-resistant strains of *S. typhi-murium* were soon isolated in 4 additional districts and became predominant in whole East Slovakia. These organisms transferred all or part of their resistance to *Escherichia coli* K12 No. 185 Nx. At the end of 1975, the occurrence of multiple drug-resistant strains of *S. typhi-murium* abruptly decreased, and in 1976 most *S. typhi-murium* strains were susceptible. In contrast, most strains of *S. agona*, *S. stanleyville* and *S. bovis-morbificans* isolated in 1976 from humans were multiple drug-resistant. These strains transferred all of their resistance to *E. coli* K12 No. 185 Nx.

COAGULASE PRODUCTION OF STAPHYLOCOCCUS AUREUS

B. ÉLIÁS

Institute of Epizootiology, University of Veterinary Science, Budapest

(i) *Staphylococcus aureus* strains (10 human, 10 bovine, 10 transitional) cultured under identical conditions for 8 hr were used to prepare cell-free coagulase preparations for titration with human and bovine plasma dilutions. All strains were tested in identical plasma batches; results were read after 4 hr. The strains exhibited coagulating activity on human/bovine plasma as follows: subsp. human: 2/0, 3/0, 5/1, 6/2, 8/0, 8/3, 9/4, 10/4, 10/6, 10/7 ($H > B$); subsp. transitional: 0/0, 3/0, 5/0, 5/4, 7/0, 7/4, 8/0, 8/3, 8/6, 9/2 ($H > B$); subsp. bovine: 3/7, 4/8, 8/8, 8/9, 4/10, 9/12, 8/13, 9/13, 6/14, 7/15 ($H < B$). (ii) Coagulase production was earliest with strains showing the highest counts in 8 hr cultures. Earliest and latest onset of coagulase production was detected after 10 and 50 min, respectively. The highest level of enzyme production was reached between the 6th and 12th hr of incubation. In mouse test the shortest time interval between inoculation and death of the animals was observed with strains of high coagulase production. (iii) If the strains were cultured in broth containing 10% human and bovine plasma for 8 hr and subjected to 3 passages, there was a 1.5–4-fold increase in homologous coagulase titres. (iv) The culture supernatant of subsp. human 76/40 coagulated 5 samples of human plasma in dilutions 4, 6, 7, 7, and 10; in 7 human plasma samples with anti-coagulase activity no detectable coagulation occurred. Similar results were obtained with 27 bovine plasma samples. (v) It is concluded that human and bovine plasma specific coagulase activity should be tested simultaneously with each strain. The human and bovine specific enzyme levels and the onset of enzyme production may differ from strain to strain. The time required for the onset of enzyme production seems to be associated with the virulence. Homologous enzyme activity increases after exposure of the cells to plasma and the enzyme pattern of the strain may reflect phylogenetic events. A precondition to coagulation seems to be the absence of an inhibitor rather than the presence of an activator.

DETERMINATION OF BACTERIAL COUNTS IN BILE

GY. DÓBIÁS and É. KROMPECHER

Postgraduate Medical School, Budapest

In 19 out of 352 patients (5.4%) not suffering from cholangitis, the bile contained 10^5 /ml or more pathogenic bacteria. Eight of the 19 patients harboured the same bacterial species in their bile and throat; 7 further patients had

anacidity or hypacidity. In 3 out of 60 samples taken from 52 patients with cholangitis, the number of bacteria was less than 10^5 /ml, whereas in 48 samples 10^5 /ml or more. The rest of the samples has not been considered because of antibiotic therapy, complete obstruction of the biliary duct, or improper handling of the specimens. None of the 29 throat specimens taken from the 52 patients yielded the bacteria cultured from bile. False negative results were attributed to contamination with gastric juice lowering the pH of the specimen to 3 or below.

MODIFIED ALKCSS MEDIUM FOR CULTURING VIBRIOS

F. KOLTA, E. SZILÁGYI and M. M. ÁDÁM

Public Health Station, Tatabánya and National Institute of Hygiene, Budapest

In search for a substitute of the expensive TCBS agar, a modified alkaline tellurite medium containing ox bile, sucrose and vancomycin was elaborated. In comparison with TCBS, the modified medium was sufficiently selective for laboratory strains of *Vibrio cholerae* and *Vibrio cholerae* var. *el-tor*. Faecal specimens from 768 patients with enteritis and healthy adults yielded 2 strains, and 80 surface water samples 42 strains of non-cholera vibrios on both kinds of medium.

METABOLIC PRODUCT ANALYSIS WITH A SIMPLIFIED GAS-LIQUID CHROMATOGRAPHIC METHOD

T. BARTHA, L. GÖNCZI and A. HOLLÓ

National Institute of Hygiene, Budapest

A simple method is described for the separation of C_1 – C_6 fatty acids and alcohols produced by anaerobic bacteria. Forty-eight hour cultures prepared in prereduced peptone–yeast extract–glucose broth are shaken with H^+ form cationic exchange resin and the aqueous supernatant is injected directly into a 2-foot long glass column packed with Porapak Q. Chromatography is performed isothermally at $193^\circ C$, and completed in 9 min. For lactic, caproic and succinic acid determination, methylated derivatives were prepared from ether extract with diazomethane, and the methylated extract was run separately on a 4-foot long glass column with Chromosorb 101 packing. The flame ionization detector was less sensitive to the methylester of lactic acid than to that of the others. Chromatograms were made from a variety of strains of commonly occurring non-sporing bacteria that had been identified by conventional

methods. Chromatographic patterns in most cases correlated well with morphological and biochemical features; the few exceptions were encountered among Gram-positive non-spore-formers. The endeavour towards accurate taxonomic determination and the need for shortening the time of diagnosis justify the metabolic product analysis in routine laboratories.

A NEW METHOD FOR TESTING THE MOTILITY OF BACTERIA

M. RODLER

Public Health Station, Szekszárd

Filter paper strips immersed in broth are inoculated with a loopful of dense bacterial suspension. After incubation in wet chamber at 37°C, the paper strips are stained with acid fuchsin. The method is suitable for the separation of flagellated and non-flagellated bacteria, for the demonstration of immune reaction between motile bacteria and the corresponding antisera and for the examination of motility-inhibition in different organisms.

MICROBIOLOGICAL STABILITY OF MEDICAL INSTRUMENTS

I. GYARMATI, K. OLÁH, Z. WOLKÓBER and P. FARKAS

Institute of Microbiology, Semmelweis University Medical School, Budapest, Ágost Schöpf-Merei Maternity Centre, Budapest and Research Institute of the Hungarian Post and Telecommunication Works, BHG, Budapest

Various instruments manufactured from 161 different kinds of plastic and rubber were tested for utilization by *Pseudomonas aeruginosa* as sole source of organic material. The specimens were attacked in 31%, moderately attacked in 47% and resistant in 22%. It has been shown that attackable instruments, as culture media for microbes, may serve as sources of iatrogenic infection upon prolonged contact with patients. The results indicate that therapeutic objects should be graded qualitatively not only in view of their toxicity and carcinogenicity, but also in terms of their microbiological stability, i.e. resistance to bacteria and fungi.

Mycology

DNA MEDIATED GENETIC CHANGES IN NEUROSPORA CRASSA

M. SCHABLIK, Á. KISS, M. SZABOLCS, J. ARADI, A. ZSINDELY,
P. ELŐDI and G. SZABÓ

*Institute of Biology, Institute of Biochemistry and Central Research Laboratory, University
Medical School, Debrecen*

Transformation of inositol requiring *Neurospora crassa* strains to inositol independence by treatment of wild type DNA (allo-DNA) was reported by SZABÓ, MISHRA and TATUM in 1972. The reproducibility of the transformation experiments was not satisfactory. In studies on the reproducibility of DNA-mediated changes we standardized our results under suitable experimental circumstances and by applying highly polymerized DNA preparations. We isolated inl^+ revertant strains without and after allo-DNA treatment. The revertants preserved their newly acquired inl^+ character in varying degree during propagation. As shown by genetic (tetrad) analysis, the allo-DNA mediated inl^+ revertants also exhibited aberrant transmission of the allo-DNA trait; the transmission of inl^+ character deviated from the expected Mendelian ratios. The two types of revertant strains were compared to establish whether any new character other than the inl^+ marker appeared in the DNA mediated transformants, e.g. changes in morphology and growth rate of the colonies, nature of perithecium production, number of asci, morphology and germination character of ascospores. It is assumed that the fragments of transforming DNA can be integrated into specific and also non-specific genome parts of the host cells or may persist in the nucleoplasm transitionally.

ISOLATION OF HIGH MOLECULAR WEIGHT DNA OF TRANSFORMING ACTIVITY FROM NEUROSPORA CRASSA

Á. KISS, M. SCHABLIK, J. ARADI, M. SZABOLCS, A. ZSINDELY and G. SZABÓ

*Institute of Biochemistry, Institute of Biology and Central Research Laboratory, University
Medical School, Debrecen*

An inositol dependent mutant (inl^-) can be transformed with DNA extracted from wild type strain (inl^+). To improve DNA isolation and to characterize DNA uptake by the mutant, the changes in extracellular DNase activity were studied during propagation. An optimum incubation time, characterized by lowest extracellular DNase activity (22–24 hr for the wild type and 18–22 hr for the mutant) was used for DNA preparation and for DNA uptake studies. To obtain high molecular weight DNA of transforming

activity and free of impurities, the procedures of MARMUR and of DUTTA were modified. The uptake of DNA by the *inl*⁻ mutant varied with the molecular weight of the DNA sample added. DNA was fractionated and purified by gel filtration on Sepharose 2B column. The product eluted at the void volume (molecular weight, 20–30 × 10⁶ daltons) was used in transforming experiments.

UPTAKE OF DNA BY *NEUROSPORA CRASSA*

J. ARADI, M. SCHABLIK, A. ZSINDELY, A. KISS, J. CSONGOR, P. ELŐDI and G. SZABÓ

Institute of Biochemistry, Institute of Biology and Central Research Laboratory, University Medical School, Debrecen

The transformation of *Neurospora crassa* strains by addition of DNA was reported by SZABÓ *et al.* in 1972. To increase the frequency of transformation, the uptake of ³²P DNA isolated from wild type *N. crassa* was studied. DNA uptake is dependent on the physiological state of the culture. Its pH, oxygenation and age influence the process. Sodium azide and EDTA inhibit the uptake. The concentration and molecular weight of DNA have a strong effect both on the rate and on the amount of incorporated DNA. The experiments allowed to define the optimum and standard conditions of DNA uptake and the results suggest that the process in *N. crassa* can be divided into two stages [1] adsorption of DNA on the cell surface and (2) biologically active stage.

STUDIES ON MYO-INOSITOL-1-PHOSPHATE SYNTHETASE FROM *NEUROSPORA CRASSA* WILD TYPE AND INOSITOL DEPENDENT MUTANT

A. ZSINDELY, M. SZABOLCS, J. ARADI, Á. KISS, M. SCHABLIK and G. SZABÓ

Institute of Biochemistry, Central Research Laboratory and Institute of Biology, University Medical School, Debrecen

For the investigation of genetic transformation induced by nucleic acids, an inositol dependent (*inl*⁻) mutant was used as recipient. Reversion of the mutant was significantly increased by DNA extracted from the wild organism (*inl*⁺). To elucidate the background of inositol dependency and the mechanism of transformation, we studied the enzyme myo-inositol-1-phosphate synthetase forming myo-inositol-1-phosphate from glucose-6-phosphate. The enzyme was purified from wild type *Neurospora crassa* by salt fractionation, gel filtration and by ion exchange chromatography. Using polyacrylamide gel electrophoresis, the product shows in addition to the main band, which is the only one having enzymatic activity, very small amounts of several accompany-

ing proteins. The molecular weight of the enzyme determined by gel filtration, and the subunit composition of the oligomeric protein are indicated. Using the same procedure, a protein was isolated from the *inl*⁻ mutant, which behaved analogously to the enzyme during fractionation, but was inactive. Preliminary data indicate that it is a defective enzyme protein.

DISCREPANCY BETWEEN THE BIOLOGICAL EFFECTIVENESS AND MEMBRANE DAMAGING ACTIVITY OF POLYENE ANTIFUNGAL ANTIBIOTICS

E. K. NOVÁK, Cs. DOBOLYI and L. KUSTÁN

National Institute of Hygiene, Budapest

As measured earlier by conductometry, 2.5 and 10 μg per ml of nystatin caused a total ion loss in cells of various yeasts in 25 and 45%, respectively. The percentage loss with flavofungin (200 $\mu\text{g}/\text{ml}$) or pimarinin (400 $\mu\text{g}/\text{ml}$) amounted to about 10%. Further investigations showed that for 75% growth inhibition (determined turbidimetrically) nystatin, pimarinin and flavofungin were needed in the following respective concentrations ($\mu\text{g}/\text{ml}$). *Candida albicans* and *Rhodotorula rubra* 2, 4, 16; *C. tropicalis* and *Saccharomyces cerevisiae* 1, 4, 16. Results for organisms tested only with nystatin and pimarinin were: *C. beverniikii*, *C. humicola*, *C. utilis* and *Sporobolomyces roseus* 1, 2; *C. reukauffii* and *C. stellatoidea* 2, 4; *Nadsonia slovacica* = *C. humicola* 2, 6; *Schizosaccharomyces octosporus* 1, 4; *Prototheca* sp. (yeast-like achloric alga) 2, 4. The above inhibiting concentrations except those of nystatin, are significantly lower than those causing ion leakage. Consequently, at least in the case of pimarinin and flavofungin, it may be assumed that the loss of ions is not the direct cause of growth inhibition.

INCIDENCE OF YEASTS IN HEALTHY AND SICK INFANTS AND YOUNG CHILDREN

K. ANDRÁSSY and I. HORVÁTH

Public Health Station, Debrecen

Infants with enterocolitis aged 0–6 months, 7–12 months, 1–3 years and 4–6 years harboured yeasts in their faeces in 30.6, 24.4, 20.0 and 18.3%, respectively. The incidence of yeasts in the faeces of healthy individuals of the same age groups was 19.5, 14.0, 15.0 and 12.2%. From throat swabs of healthy children, *Candida albicans* was cultured in 19.9%, other yeasts in 10.3%. Children with spastic bronchitis and bronchial asthma carried these organisms in 50.4 and 15.8%.

MICROSPORUM FELINEUM RESPONSIBLE FOR A FAMILY INFECTION
OF DERMATOPHYTOSIS

T. GAÁL, J. GALGÓCZY and Z. HORVÁTH

*Institute of Medicine, University Veterinary School, Budapest and National Institute of Hygiene
Budapest*

A family infection by *Microsporum felineum* was traced to a stray cat. Mycotic changes appeared in two men and three animals. Local treatment was effective in humans and animals in two weeks. It is concluded that mycological examination may reveal the presence of species seldom encountered in Hungary before. Recent isolates and a laboratory strain of *M. felineum* were characterized by the finding that the appearance of macroconidia was promoted on traditional rice-agar or on Sabouraud-glucose agar containing 3% NaCl. The so-called "free-microcultures" were the most suitable means of observation. Results obtained for the ontogeny of the macroconidia of this species agree with previous observations on dermatophytes, showing that macroconidia are characterized by holoblastic conidium ontogeny, primary septum and detaching cell. Staining of detached macroconidia starts at the basal and apical areas.

EFFECT OF ZEARALENONE FUSARIUM TOXIN ON THE PROSTATE
GLAND OF SWINE

M. PLYUSIK

Veterinary Research Institute of the Hungarian Academy of Sciences, Budapest

Miniature male swines weighing 18.5–20 kg were fed continuously for two weeks with feed containing 100 ppm zearalenone (F2 toxin). In addition to a considerable diminution of the testis, epididymis, vesicular glands, and besides a moderate diminution of Cowper's glands, enlargement of the prostate was observed. Since thermostable zearalenone may be present not only in corn, but also in wheat, further investigations are necessary to establish the possible health-impairing role of this mycotoxin.

TOXINS PRODUCED BY *PENICILLIUM*

G. SELLYEY, E. and R. SZAILER

Central Veterinary Institute, Budapest

Patulin, an antibiotic lactone metabolite of several species of *Penicillium* and *Aspergillus*, exerts toxic effects if produced in contaminated feedstuffs during storage. For detecting the formation of patulin in time, experiments

were made with three *Penicillium* and one *Aspergillus* strains by artificial contamination of culture medium and feedstuff. The samples were extracted with acetonitrile-hexane, then, after evaporation to dryness in steam bath, dissolved in 0.5 ml chloroform. Chromatography was carried out in Kieselgel 60 HF₂₅₄₊₃₆₆ layer. In benzene : methanol : acetic acid (90 : 5 : 5) solvent system, patulin appeared at R_F 0.34. For the detection of patulin the phenylhydrazone formation reaction was the most sensitive. The reliability of the analytical method was checked by determining the recovery per cent for five feedstuff batches.

MICROSOMAL BIOTRANSFORMATION OF TRICHOHECENE MYCOTOXINS

I. UENO and M. OHTA

Institute of Microbial Chemistry, Faculty of Pharmaceutical Sciences, Tokyo University of Sciences, Tokyo, Japan

More than thirty kinds of 12,13-epoxytrichothecene mycotoxins have been isolated from *Fusarium*, *Myrothecium*, *Stachybotrys* and other organism. Mycological and toxicological surveys revealed that some of these mycotoxins are closely related to the natural occurrence of alimentary intoxications of humans and farm animals. The trichothecene compounds, which differ from each other in substituents at five positions in the nuclei, have been classified into two groups: type (A) and type (B). In the present work, the fate and metabolism of trichothecenes have been studied. Tracer experiments with T-2 toxin and fusarenon-X revealed that these mycotoxins are excreted into the faeces of rats and mice as a deacetylated product such as HT-2 toxin, and nivalenol. TLC and GLC analysis of the *in vitro* metabolites indicated that T-2 toxin was biotransformed into one metabolite, HT-2 toxin, by 9000 g supernatant of the liver and this transforming activity was mostly located in the microsomal fraction. Activity was highest in the liver of the rabbit, followed by guinea pig, human, rat and mouse. Hydrolytic activity was found in the liver, kidneys, spleen and brain. Since this type of transformation was inhibited by eserine and diisopropylfluorophosphate, cholinesterase catalyses the reaction. As for the structure-activity relationship of "trichothecene esterase" of the microsomes, T-2 toxin, diacetoxyscirpenol and fusarenon-X were deacetylated at C-4 (R-2) into the corresponding products such as HT-2 toxin, monoacetoxyscirpenol and nivalenol. No hydrolysis occurred with acetyl-t-2 toxin and neosolaniol. These findings support the assumption that the acetyl group at C-4 (R-2) is stereospecifically eliminated by the microsomal esterase(s) and this hydrolysis does not take place when the hydroxyl group at C-3 (R-1) is acetylated and the C-8 (R-5) is occupied

by a hydroxyl group. These enzymatic approaches to the metabolism of trichothecenes represent a key for epidemiological survey of natural occurrence of pollution of humans and farm animals by trichothecene mycotoxins.

BIOLOGICAL ACTIVITY OF VARIOUS DERIVATIVES OF ZEARALENONE

C. J. MIROCHA

Department of Plant Pathology, University of Minnesota, St. Paul, Minnesota, U.S.A.

Fusarium roseum, while colonizing maize, produces an estrogenic metabolite called zearalenone: 2,4-dihydroxy-6 (10-hydroxy-6-oxo-trans-undecenyl) benzoic acid u-lactone. This estrogen effects the reproductive organs of swine, mice, rats, guinea pigs, rabbits, chicks, lambs, turkeys and monkeys. Notable is its sex-regulating activity of perithecium development in *F. roseum* (stimulating at low concentrations and inhibiting at high concentrations). Chemically derived derivatives that are active in *Fusarium* are usually active in animals and *vice versa*. The products of metabolism of zearalenone by *Fusarium* are: (i) the two isomers of 8'-hydroxyzearalenone; (ii) 6'8'-dihydroxyzearalenol; (iii) 6-carboxy-pentyl, beta-resorcilic acid lactone and (iv) the aldehyde of 6-carboxypentyl-beta resorcilic acid lactone. The above metabolites are inactive in rat uterine tests. The naturally occurring trans isomer of zearalenone could easily be converted to the *cis* isomer by irradiation with ultraviolet. Contrary to expectations, the *cis* isomer was 4-5 times more active in the rat uterine test. The trans-zearalenol was similarly converted to the *cis* isomer but no difference in activity on the rat was noted. When both the *cis* and *trans* isomers of zearalenone were tested for their respective activity in regulating the production of perithecia and ascospore development in *F. roseum*, no difference was found like that encountered in the fat. The activity of the respective *cis* and *trans* isomers of zearalenone and zearalenol will be tested in sexually immature female swine.

INDEX

<i>Molnár, J., Mándi, Y., Holland, I. B., Schneider, Gy.:</i> Antibacterial Effect, Plasmid Curing Activity and Chemical Structure of Some Tricyclic Compounds	1
<i>Dhikidze, E. K., Kavtaradze, K. N., Krilova, R. I., Rauss, K., Kétyi, I., Vertényi, A.:</i> Oral Immunization of Monkeys with Polyvalent Dysentery Vaccine	7
<i>Gergely, L., Czeglédy, J., Szalka, A., Vácz, L., Binder, L.:</i> Epstein-Barr Virus and Cytomegalovirus Antibodies in Infectious Mononucleosis	13
<i>Boldogh, I., Gönczöl, É., Vácz, L.:</i> Cytomegalovirus-Induced Membrane Antigens in Productively Infected Cells	21
<i>Sipka, S., Boldogh, I., Szilágyi, T.:</i> Macrophage Disappearance Reaction in <i>Rana esculenta</i> Induced by Specific Antigen and Rabbit Lymphokines	29
<i>Singh, A., Mohan, K. S., Sethi, S. K., Vadehra, D. V.:</i> Cross-Neutralization Reactions of <i>Escherichia coli</i> and <i>Vibrio cholerae</i> Enterotoxins as Studied by Rabbit Skin Inoculation Test	33
<i>Seventh Congress of the Hungarian Society of Microbiology, Budapest, September 6-9, 1976.</i>	
Abstracts of papers	41
Plenary Session	43
Immunology	48
Interaction of pesticides and microorganisms	57
Virology	63
Bacterial genetics	80
Bacteriology	92
Mycology	101

Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója

Műszaki szerkesztő: Zacsik Annamária

A kézirat nyomdába érkezett: 1977. I. 11. — Terjedelem: 9,8 (A5) ív, 8 ábra

77.4033 Akadémiai Nyomda, Budapest — Felelős vezető: Bernát György

Die *Acta Microbiologica* veröffentlichen Abhandlungen aus dem Bereiche der Mikrobiologie in deutscher, englischer, französischer und russischer Sprache.

Die *Acta Microbiologica* erscheinen in Heften wechselnden Umfanges. Mehrere Hefte bilden einen Band.

Die zur Veröffentlichung bestimmten Manuskripte sind an folgende Adresse zu senden:

Acta Microbiologica, Staatliches Institut für Hygiene, H-1966 Budapest, P. O. B. 64, Ungarn

An die gleiche Anschrift ist auch jede für die Redaktion bestimmte Korrespondenz zu richten. Abonnementspreis pro Band: \$ 32.00.

Bestellbar bei dem Außenhandels-Unternehmen «Kultúra» (H-1389 Budapest 62, P. O. B. 149, Bankkonto Nr. 218-10990) oder bei seinen Auslandsvertretungen und Kommissionären.

Les *Acta Microbiologica* paraissent en français, allemand, anglais et russe et publient des travaux du domaine de la microbiologie.

Les *Acta Microbiologica* sont publiés sous forme des fascicules qui seront réunis en volumes.

On est prié d'envoyer les manuscrits destinés à la rédaction à l'adresse suivante:

Acta Microbiologica, Institut National d'Hygiène Publique, H-1966 Budapest, P. O. B. 64, Hongrie

Toute correspondance avec la rédaction doit être envoyée à cette même adresse.

Le prix de l'abonnement est de \$ 32.00 par volume.

On peut s'abonner à l'Entreprise pour le Commerce Extérieur «Kultúra» (H-1389 Budapest 62, P. O. B. 149, Compte courant No. 218-10990) ou à l'étranger chez tous les représentants ou dépositaires.

«*Acta Microbiologica*» публикуют трактаты из области микробиологии на русском, немецком, английском и французском языках.

«*Acta Microbiologica*» выходят отдельными выпусками разного объема. Несколько выпусков составляют один том.

Предназначенные для публикации рукописи следует направлять по адресу:

Acta Microbiologica, Институт Здравоохранения, H-1966 Budapest, P. O. B. 64, Венгрия

По этому же адресу направлять всякую корреспонденцию для редакции.

Подписания цена — \$ 32.00 за том.

Заказы принимает предприятие по внешней торговле «Kultúra» (H-1389 Budapest 62, P. O. B. 149, Текущий счет № 218-10990), или его заграничные представительства и уполномоченные.

Reviews of the Hungarian Academy of Sciences are obtainable
at the following addresses:

AUSTRALIA

C.B.D. LIBRARY AND SUBSCRIPTION SERVICE
Box 4886, G.P.O., Sydney N.S.W. 2001
COSMOS BOOKSHOP, 145 Ackland Street, St.
Kilda (Melbourne), Victoria 3182

AUSTRIA

GLOBUS, Höchstädtplatz 3, 1200 Wien XX

BELGIUM

OFFICE INTERNATIONAL DE LIBRAIRIE, 30
Avenue Marnix, 1050 Bruxelles
LIBRAIRIE DU MONDE ENTIER, 162 Rue du
Midi, 1000 Bruxelles

BULGARIA

HEMUS, Bulvar Ruszki 6, Sofia

CANADA

PANNONIA BOOKS, P.O. Box 1017, Postal Sta-
tion "B", Toronto, Ontario M5T 2T8

CHINA

CNPICOR, Periodical Department, P.O. Box 50,
Peking

CZECHOSLOVAKIA

MAD'ARSKÁ KULTURA, Národní třída 22,
115 66 Praha

PNS DOVOZ TISKU, Vinohradská 46, Praha 2

PNS DOVOZ TLAČE, Bratislava 2

DENMARK

EJNAR MUNKSGAARD, Norregade 6, 1165
Copenhagen

FINLAND

AKATEEMINEN KIRJAKAUPPA, P.O. Box 128,
SF-00101 Helsinki 10

FRANCE

EUROPERIODIQUES S. A., 31 Avenue de Ver-
sailles, 78170 La Celle St. Cloud

LIBRAIRIE LAVOISIER, 11 rue Lavoisier, 75008
Paris

OFFICE INTERNATIONAL DE DOCUMENTA-
TION ET LIBRAIRIE, 48 rue Gay Lussac, 75240
Paris Cedex 05

GERMAN DEMOCRATIC REPUBLIC

HAUS DER UNGARISCHEN KULTUR, Karl-
Liebknecht-Strasse 9, DDR-102 Berlin

DEUTSCHE POST ZEITUNGSVERTRIEBSAMT,
Strasse der Pariser Kommune 3-4, DDR-104 Berlin

GERMAN FEDERAL REPUBLIC

KUNST UND WISSEN ERICH BIEBER, Postfach
46, 7000 Stuttgart 1

GREAT BRITAIN

BLACKWELL'S PERIODICALS DIVISION, Hythe
Bridge Street, Oxford OX1 2EU

BUMPUS, HALDANE AND MAXWELL LTD.,
Cowper Works, Olney, Bucks MK46 4BN

COLLET'S HOLDINGS LTD., Denington Estate,
Wellingborough, Northants NN8 2QT

WM. DAWSON AND SONS LTD., Cannon House,
Folkestone, Kent CT1 5EE

H. K. LEWIS AND CO., 136 Gower Street, London
WC1E 6BS

GREECE

KOSTARAKIS BROTHERS, International Book-
sellers, 2 Hippokratous Street, Athens-143

HOLLAND

MEULENHOF-BRUNA B.V., Beulingstraat 2,
Amsterdam

MARTINUS NIJHOFF B.V., Lange Voorhout
9-11, Den Haag

SWETS SUBSCRIPTION SERVICE, 347b Heere-
weg, Lisse

INDIA

ALLIED PUBLISHING PRIVATE LTD., 13/14
Asaf Ali Road, New Delhi 110001

150 B-6 Mount Road, Madras 600002

INTERNATIONAL BOOK HOUSE PVT. LTD.,
Madame Cama Road, Bombay 400039

THE STATE TRADING CORPORATION OF
INDIA LTS., Books Import Division, Chandralok,
36 Janpath, New Delhi 110001

ITALY

EUGENIO CARLUCCI, P.O. Box 252, 70100 Bari
INTERSCIENTIA, Via Mazzè 28, 10149 Torino

LIBRERIA COMMISSIONARIA SANSONI, Via
Lamarmora 45, 50121 Firenze

SANTO VANASIA, Via M. Macchi 58, 20124
Milano

D. E. A., Via Lima 28, 00198 Roma

JAPAN

KINOKUNIYA BOOK-STORE CO. LTD., 17-7

Shinjuku-ku 3 chome, Shinjuku-ku, Tokyo 160-91

MARUZEN COMPANY LTD., Book Department,
P.O. Box 5050 Tokyo International, Tokyo 100-31

NAUKA LTD. IMPORT DEPARTMENT, 2-30-19
Minami Ikebukuro, Toshima-ku, Tokyo 171

KOREA

CHULPANMUL, Phenjan

NORWAY

TANUM-CAMMERMEYER, Karl Johansgatan
41-43, 1000 Oslo

POLAND

WĘGIERSKI INSTYTUT KULTURY, Marszał-
kowska 80, Warszawa

CKPI W ul. Towarowa 28 00-958 Warsaw

ROUMANIA

D. E. P., București

ROMLIBRI, Str. Biserica Amzei 7, București

SOVIET UNION

SOYUZPETCHATY — IMPORT, Moscow

and the post offices in each town

MEZHDUNARODNAYA KNIGA, Moscow G-200

SPAIN

DIAZ DE SANTOS, Lagasca 95, Madrid 6

SWEDEN

ALMQVIST AND WIKSELL, Gamla Brogatan 26,
S-101 20 Stockholm

GUMPERTS UNIVERSITETSBOKHANDEL AB,
Box 346, 401 25 Göteborg 1

SWITZERLAND

KARGER LIBRI AG, Petersgraben 31, 4011 Basel

USA

EBSCO SUBSCRIPTION SERVICES, P.O. Box
1943, Birmingham, Alabama 35201

F. W. FAXON COMPANY, INC., 15 Southwest
Park, Westwood, Mass. 02090

THE MOORE-COTTRELL SUBSCRIPTION

AGENCIES, North Cohocton, N. Y. 14868

READ-MORE PUBLICATIONS, INC., 140 Cedar
Street, New York, N. Y. 10006

STECHERT-MACMILLAN, INC., 7250 Westfield
Avenue, Pennsylvania N. J. 08110

VIETNAM

XUNHASABA, 32, Hai Ba Trung, Hanoi

YUGOSLAVIA

JUGOSLAVENSKA KNJIGA, Terazije 27, Beograd
FORUM, Vojvode Mišića 1, 21000 Novi Sad

ACTA MICROBIOLOGICA

ACADEMIAE SCIENTIARUM
HUNGARICAE

ADIUVANTIBUS

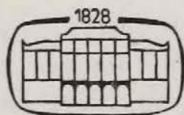
L. ALFÖLDI, I. BÉLÁDI, I. DÖMÖK, E. FARKAS, G. FARKAS,
L. FERENCZY, I. FÖLDES, I. HORVÁTH, I. JOÓ, I. KÉTYI,
I. KESZTYŰS, B. LÁNYI, I. NÁSZ, I. M. SZABÓ, L. VÁCZI,
J. WEISZFEILER

REDIGIT

G. IVÁNOVICS

TOMUS XXIV

FASCICULUS 2



AKADÉMIAI KIADÓ, BUDAPEST

1977

ACTA MICROBIOLOGICA

A MAGYAR TUDOMÁNYOS AKADÉMIA MIKROBIOLÓGIAI KÖZLEMÉNYEI

KIADÓHIVATAL: 1954 BUDAPEST, ALKOTMÁNY UTCA 21.

A Szerkesztő bizottság elnöke :
IVÁNOVICS GYÖRGY
akadémikus

Főszerkesztő :
LÁNYI BÉLA

Az *Acta Microbiologica* német, angol, francia és orosz nyelven közöl értekezéseket a mikrobiológia tárgyköréből.

Az *Acta Microbiologica* változó terjedelmű füzetekben jelenik meg. Több füzet alkot egy kötetet.

A közlésre szánt kéziratok a következő címre küldendők:

Acta Microbiologica, Országos Közegészségügyi Intézet, 1966 Budapest, Pf. 64.

Ugyanerre a címre küldendő minden szerkesztőségi levelezés.

Megrendelhető a belföld számára az Akadémiai Kiadónál (1363 Budapest Pf. 24 Bankszámla 215-11448), a külföld számára pedig a „Kultúra” Külkereskedelmi Vállalatnál (1389 Budapest 62, Pf. 149, Bankszámla: 218-10990) vagy annak külföldi képviselőinél és bizományosainál.

The *Acta Microbiologica* publish papers on microbiological subjects in English, German, French and Russian.

The *Acta Microbiologica* appear in parts of varying size, making up volumes.

Manuscripts should be addressed to

Acta Microbiologica, National Institute of Hygiene, H-1966 Budapest, P. O. B. 64, Hungary

Correspondence with the editors should be sent to the same address.

The rate of subscription is \$ 32.00 a volume.

Orders may be placed with “Kultúra” Foreign Trade Company (H-1389 Budapest 62, P. O. B. 149, Account No. 218-10990) or with representatives abroad.

MAGYAR
TUDOMÁNYOS AKADÉMIA
KÖNYVTÁRA

TRANSFER OF ERYTHROMYCIN RESISTANCE IN STAPHYLOCOCCUS AUREUS

JUDITH LANTOS

Public Health Station, Szeged

(Received June 1, 1976)

In the course of an outbreak of enteritis and conjunctivitis, *Staphylococcus aureus* was isolated from newborn infants. Strains cultured at a later phase of the outbreak differed from those found at the beginning in being resistant to several antibiotics, showing resistance to typing phages and releasing phages of the same lysis spectrum (10^9 p.f.u./ml after heating at 56 °C for 2 min). Transduction experiments with a strain and its cell-free lysate showed that inducible erythromycin resistance was transferable to strains isolated at the beginning of the outbreak and to laboratory strains. Plasmid origin of resistance was confirmed by (i) high transduction frequency; (ii) transduction to RN981 *rec*⁻ mutants; (iii) kinetics of transduction; (iv) elimination of resistance. Mixed culture experiments yielded transductants at high frequency with resistance to erythromycin, streptomycin and tetracycline.

Epidemiological observations and resistance transfer experiments with phages have shown that the development of antibiotic resistance in most bacteria is associated with plasmids. NOVICK [1] was the first to confirm by transduction analysis the plasmid origin of penicillinase production and showed that this plasmid was responsible for other properties, for example, erythromycin resistance. PATTEE and BALDWIN [2], then WEAVER and PATTEE [3] reported on the transduction of resistance to macrolid antibiotics in staphylococci. They confirmed the finding of GARROD [4] that erythromycin resistance can be induced and that erythromycin at 0.01 µg/ml concentration gives rise to resistance to oleandomycin and spiramycin. HASHIMOTO *et al.* [5] described similar results. In 1969 WEISBLUM and DEMOHN [6] reviewed the studies on inducible erythromycin resistance. The plasmid character of erythromycin resistance was not known until 1972 when BRONSON and PATTEE [7] transduced erythromycin and oleandomycin resistance to mutants of a *S. aureus* strain and showed that the same locus was involved for both antibiotics. The locus was inducible for erythromycin marker and constitutive for oleandomycin marker; its plasmid nature, however, was not proven. DORNBUSCH and DAHLSTRÖM [8] showed that inducible erythromycin resistance was bound to plasmids. This observation was confirmed by NOVICK and BOUANCHAUD [9], LACEY [10] and GRINSTED and LACEY [11]. The plasmid DNA in covalently closed circular form responsible for erythromycin resistance has not yet been isolated.

The present paper reports on examination of *Staphylococcus aureus* strains isolated in the course of an enteritis and conjunctivitis outbreak in a newborn ward. From antibiotic resistance, phage pattern and lysogenicity examinations it was assumed that plasmid resistance transfer had occurred.

Materials and methods

Strains. Out of 58 *S. aureus* strains 13 were isolated at the beginning of the outbreak from infants and nurses; these cultures showed phage pattern 53/83A/77 and 7 of them were resistant only to penicillin, while the rest to penicillin, tetracycline and chloramphenicol. Forty-five strains isolated later during the outbreak were uniformly resistant to erythromycin, streptomycin, tetracycline, methicillin and penicillin and were untypable (lysis with phages 77 and 88 was observed only at $100 \times$ RTD).

One strain from each culture was selected as representative (Table I). Recipient strains 560 and 612 were isolated from a nurse and from an infant respectively. Antibiotic sensitive strains belonging to different phage types not associated with the outbreak were also used as recipients: strain 2919 obtained from the Institute of Experimental Epidemiology, Wernigerode, GDR, was, to our knowledge, not lysogenic; strain RN 981 received from Dr. E. H. ASHESHOV, Central Public Health Laboratory, Colindale, London, was a recombination-defective mutant of strain Ps 47 not producing stable chromosomal mutants. Multiple resistant strain 780, isolated from the outbreak, was used as donor. The titre of phages spontaneously released from this strain was 10^6 p.f.u./ml; after heat treatment at 56°C for 2 hr the titre of the lysate was 10^9 p.f.u./ml. Transduction was performed with the cell-free lysate of this phage (ϕ 780) as in this manner propagation on an intermediary strain could be omitted and a possible modification or restriction of the phage avoided. Phage ϕ 780 showed a broad lysis spectrum on standard propagating strains and lysed cultures isolated at the beginning of the outbreak. Titration of this phage was performed on strain 1217. Mixed culture transduction was carried out with penicillin and chloramphenicol resistant strain 807 not associated with the outbreak; the strain was sensitive to phage ϕ 780.

Culture medium. Broth: Bacto peptone (Difco), 10 g; Lab-Lemco meat extract (Oxoid), 10 g; NaCl, 5 g; water, 1000 ml; pH 7.4; 121°C , 30 min. Solid medium was prepared from broth by adding 11 g No. 1 agar (Oxoid) per 1000 ml. For selecting transductants, erythromycin lactobionate (25 $\mu\text{g}/\text{ml}$), tetracycline (20 $\mu\text{g}/\text{ml}$), streptomycin (40 $\mu\text{g}/\text{ml}$), for mixed cultures erythromycin lactobionate (25 $\mu\text{g}/\text{ml}$) + chloramphenicol (20 $\mu\text{g}/\text{ml}$) were added prior to pouring plates. For phage typing and transduction the media were supplemented with calcium chloride (200 $\mu\text{g}/\text{ml}$).

Antibiotic sensitivity was tested by the use of Resistest disks (Institute for Serobacteriological Production and Research Human, Budapest). Part of the colonies was tested by tube dilution technique for minimal inhibitory concentration (MIC) using an inoculum of approximately 10^5 cells and incubation at 37°C for 24 hr.

Elimination of antibiotic resistance was performed by culturing the multiple resistant strain in broth containing acriflavine (25 $\mu\text{g}/\text{ml}$), ethidium bromide (20 $\mu\text{g}/\text{ml}$) and chlorpromazine (25 $\mu\text{g}/\text{ml}$) [12] at 37°C for 24–48 hr, at 43°C for 48 hr and at room temperature for 8 weeks. Dilution of the cultures were streaked at 0.1 ml amounts on agar plates; after 24 hr incubation the colonies were replicated onto plates containing antibiotics. Colonies having lost their resistance were subcultured several times and checked by the disk method.

Transduction was made as described by ASHESHOV [13] in the presence of calcium chloride (200 $\mu\text{g}/\text{ml}$) and erythromycin (0.1 $\mu\text{g}/\text{ml}$). Agar plates containing antibiotics were used for selection. The mixed culture (recipient + donor) was layered on agar plates at 0.15 ml aliquots containing 50 μg calcium chloride and 1 μg erythromycin. After incubation at 37°C for 18 hr the culture was suspended in 5 ml saline and streaked at different dilutions on plates containing erythromycin (25 $\mu\text{g}/\text{ml}$) and chloramphenicol (20 $\mu\text{g}/\text{ml}$). Sterility of transducing phage and presence of mutants of the recipient and donor strains were checked on control plates. In each experiment about 10 colonies were examined by phage typing and resistance testing. Frequency of transduction was expressed as a ratio of the number of transductants and p.f.u. of the transducing phage; for mixed cultures the ratio of the number of colony forming units of the donor strain was calculated. Survival of the transducing phage after exposure to UV light was examined with a 220 V Medicor bulb at 30 cm distance.

Table I

Phenotype of strains used in the experiments

Strain		Antibiotic resistance spectrum	Phage pattern		Minimum inhibitory concentration	
		P M E O L C N S T	RTD	100×RTD	penicillin $\mu\text{g/ml}$	erythromycin $\mu\text{g/ml}$
Recipient	560	R S S S S S S S S	53/83A	77	3×10^3	1
	612	R S S S S S S S R	53/83A	88	3×10^3	1
	2919	S S S S S S S S S	6/47/53/54/75/81		1	1
	1217	S S S S S S S S S	phage group I, II, III		1	1
	RN 981	S S S S S S S S S	47/53/54/75/77/84/85		1	1
Donor	807	R S S S S R S S S	53/83A/88		3×10^3	1
	780	R R R S S S S R R	n. t.	77/88	3×10^3	1×10^5

P = penicillin

M = methicillin

E = erythromycin

O = oleandomycin

C = chloramphenicol

N = neomycin

S = streptomycin

T = tetracycline

L = lincomycin

R = resistant

S = sensitive

Results

Elimination of erythromycin resistance is shown in Table II. Erythromycin sensitive colonies obtained after chlorpromazine treatment became sensitive to methicillin and streptomycin, but retained their penicillin and tetracycline resistance. Mixed cultures left to stand at room temperature yielded after curing, with one exception, colonies sensitive to all the above antibiotics.

Transduction analysis of erythromycin resistance. Only erythromycin resistance could be transferred at high frequency. The results for one donor strain and various recipient strains are summarized in Table III. By raising

Table II

Elimination of antibiotic resistance from multiple resistant S. aureus strain 780

Mode of curing	No. of colonies tested	No. of sensitive colonies			
		erythro-mycin	strepto-mycin	tetra-cyclin	methi-cillin
Acridflavine (25 $\mu\text{g/ml}$)	1000	0	0	0	0
Ethidium bromide (20 $\mu\text{g/ml}$)	1977	0	0	0	0
Chlorpromazine (25 $\mu\text{g/ml}$)	1834	12	12	0	12
Culturing at 43 °C for 48 hr	2934	0	0	0	0
Standing at room temperature for 8 hr	1965	7	6	6	7

Table III

Frequency of erythromycin resistance transduction in systems of donor strain 780 and various recipient strains

Recipient strain	Multiplicity	Time		Erythromycin induction	Frequency of transduction
		1 hr	20 hr		
560	0.5	+		—	1.10^{-8}
560	0.5		+	—	4.10^{-8}
560	0.7	+		+	1.10^{-7}
560	0.7		+	+	3.10^{-7}
560	1		+	+	5.10^{-6}
612	1		+	+	1.10^{-3}
2919	1		+	+	5.10^{-6}
1217	1		+	+	1.10^{-6}
RN 981 <i>rec</i> ⁻	0.1		+	+	1.10^{-7}
RN 981 <i>rec</i> ⁻	1		+	+	8.10^{-7}

multiplicity and prolonging the time of incubation, the frequency of transduction increased. Resistance was inducible, as at concentrations lower than MIC the number of transductants increased 10 to 100 times. This was confirmed by the following experiment. Onto plates overlaid with cultures of the donor strain and erythromycin resistant transductants, one erythromycin disk and, at 20 mm distance from it, one oleandomycin and one lincomycin disk were placed. The latter disks inhibited growth only at sites falling outside the diffusion range of erythromycin.

Kinetics of transduction. The transducing phage was subjected to increasing doses of UV irradiation. Figure 1 shows the effect of irradiation on transduction of inducible erythromycin resistance for heat-induced cell-free lysates of recipient strain 560 (penicillin resistant) and donor strain 780 (multiple resistant). The decrease in percentage of surviving phage with the increase of UV dose and the logarithm of transduction frequency after 1 hr and 24 hr, calculated from the number of erythromycin resistant transductants, are presented. The frequency curve shows an exponential decrease characteristic of the transduction of plasmid genes. The plasmid origin was supported also by transduction to recipient RN981, as this strain fails to give stable transductants with chromosomal genes.

Properties of the transductants are shown in Table IV. Strains having acquired inducible erythromycin resistance were almost as resistant to this antibiotic as the donor strain and showed no change in phage pattern.

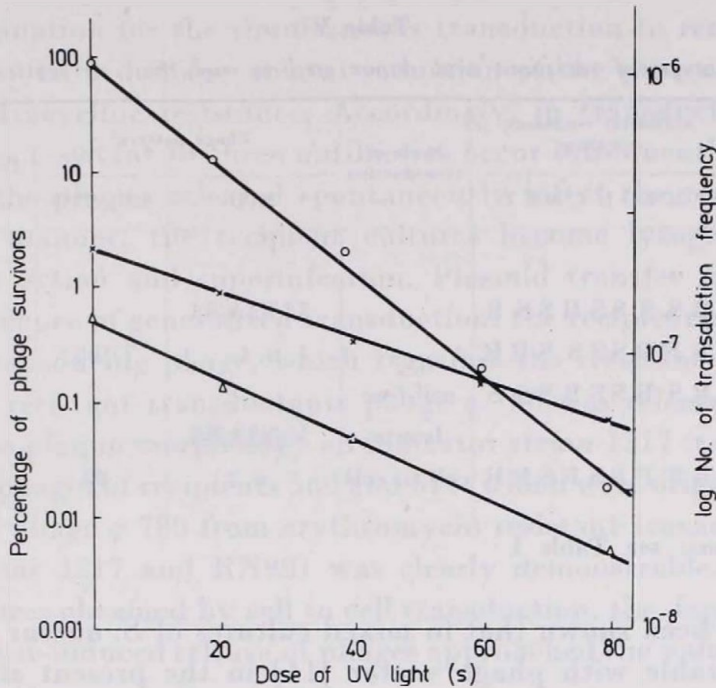


Fig. 1. Transduction frequency of inducible erythromycin resistance as a function of the time of UV irradiation of transducing phage. Donor: cell-free lysate of multiple resistant *S. aureus* strain 780; recipient: *S. aureus* strain 560 pen⁺. ○ Number of survivors after UV irradiation; Δ transduction of erythromycin resistance after 1 hr; × transduction of erythromycin resistance after 20 hr

Table IV

Phenotype of original and erythromycin resistant transductants

Strain	Antibiotic resistance spectrum	Phage pattern		Minimum inhibitory concentration, erythromycin µg/ml
	P M E O L C N S T	RTD	100×RTD	
560	R S S S S S S S S	53/83A	77	1
560	R S R S S S S S S	53/83A	77	2.5×10^4
612	R S S S S S S S R	53/83A/88		1
612	R S R S S S S S R	53/88	83A	2.5×10^4
2919	S S S S S S S S S	6/47/54		1
2919	S S R S S S S S S	6/47/54/75/81		2.5×10^4
RN 981	S S S S S S S S S	47/53/54/75/77/84/85		1
RN 981	S S R S S S S S S	47/53/54/75/77/84/85		2.5×10^4
1217	S S S S S S S S S	phage group I, II, III		1
1217	S S R S S S S S S	phage group I, II, III		1×10^4

Abbreviations: see Table I

Table V
Phenotype of recipient and donor strains and their derivatives

Strain		Antibiotic resistance spectrum	Mode of transduction	Phage pattern		Frequency of transduction
		P M E O L C N S T		RTD	100×RTD	
Recipi- ent	807	R S S S S R S S S	cell-free lysate	53/83A/88	77/88	2×10^{-4}
	780	R R R S S S S R R		n. t.		
Donor	807-31	R S R S S R S S S	cell to cell	53/83A/88	88	$5 \times 10^{-5} - 2 \times 10^{-6}$
	807-5	R S R S S R S R R		n. t.		

Abbreviations: see Table 1

As it had been shown that in mixed cultures of *S. aureus* plasmid resistance is transferable with phage vector [14] in the present studies a model donor-recipient system was developed in which recipient strain 807 was penicillin and chloramphenicol resistant; the donor strain (780) was multiple resistant (Table V).

Erythromycin resistance was transferred to recipient strain 807 both with cell-free lysates of strain 780 and with cell to cell transduction. Cell-free lysates induced only erythromycin resistance, whereas in mixed cultures a high transduction frequency to erythromycin + streptomycin + tetracycline resistance was obtained.

Discussion

The rapid spread of multiple resistant staphylococci during the outbreak suggested a plasmid-mediated transfer of resistance *in vivo*. Laboratory experiments showed that the heat-induced transducing phage transferred only erythromycin resistance. The plasmid origin was confirmed by (1) high transduction frequency; (2) transduction into *rec*⁻ mutant; (3) kinetics of transduction and (4) curing of resistance. These conditions, however, are too artificial to take place *in vivo*.

Mixed culture experiments yielded multiple resistant strains at high frequency.

As the simultaneous transduction of three different resistance genes at a frequency of the observed order (Table V) seems highly improbable, it may be assumed that the chloramphenicol resistance gene of strain 807 appeared in the multiple resistant strain. Since from strain 807 no phage acting upon strain 780 was released, the action of a defective phage as transfer vector may be suggested.

An explanation for the simultaneous transduction to resistance to three antibiotics may be a damage at heat-induction of the gene carrying streptomycin and tetracycline resistance. Accordingly, in transduction with phage lysates colonies resistant to three antibiotics occur infrequently. In cell to cell transduction the phages released spontaneously infect the recipient strain in a continuous manner; the recipient cultures become lysogenized and thus survive phage action and superinfection. Plasmid transfer may be assumed to be a consequence of generalized transduction: the recipient strain is lysogenized by the transducing phage, which transfers the resistance plasmid. From erythromycin resistant transductants phage φ 780 was released. On the basis of characteristic plaque morphology on indicator strain 1217 it was distinguishable from the phages of recipients 560 and 612 (which were originally lysogenic). The release of phage φ 780 from erythromycin resistant transductants of non-lysogenic strains 1217 and RN981 was clearly demonstrable. From multiple resistant cultures obtained by cell to cell transduction, the degree of the spontaneous and heat-induced release of phages approached the values for the donor strain.

CHOPRA *et al.* [15] described plasmids responsible for resistance to more than one antibiotic. According to LACEY [14] the transduction frequency depends on the particular plasmid, donor, prophage present in the donor, cultural conditions and the ratio of donor and recipient. In appropriate fresh isolates selected at random, the frequency of plasmid transfer after incubation overnight is 10^{-4} – 10^{-8} . In view of these findings, the assumption that resistance transfer occurred *in vivo* does not seem improbable.

Acknowledgement. The author wishes to thank Mrs. E. FOGARASI for skilled technical assistance.

REFERENCES

1. NOVICK, R. P.: J. gen. Microbiol. **33**, 121 (1963).
2. PATTEE, P. A., BALDWIN, J. E.: J. Bact. **84**, 1049 (1962).
3. WEAWER, J. R., PATTEE, P. A.: J. Bact. **88**, 574 (1964).
4. GARROD, L. P.: Brit. med. J. **2**, 57 (1957).
5. HASHIMOTO, H., OSHIMA, H., MITSUHASHI, S.: Jap. J. Microbiol. **39**, 321 (1968).
6. WEISBLUM, B., DEMOHN, V.: J. Bact. **98**, 447 (1969).
7. BRONSON, D. L., PATTEE, P. A.: Canad. J. Microbiol. **18**, 429 (1972).
8. DORNBUSH, K., DAHLSTRÖM, A.: Acta path. microbiol. scand. B. **81**, 497 (1973).
9. NOVICK, R. P., BOUANCHAUD, D.: Ann. N. Y. Acad. Sci. **182**, 279 (1971).
10. LACEY, R. W.: J. med. Microbiol. **5**, 497 (1972).
11. GRINSTED, J., LACEY, R. W.: J. med. Microbiol. **6**, 351 (1973).
12. MÁNDI, Y., MOLNÁR, J., HOLLAND, I. B., BÉLÁDI, I.: Genet. Res. **26**, 109 (1976).
13. ASHESHOV, E. H.: J. gen. Microbiol. **59**, 289 (1969).
14. LACEY, R. W.: Bact. Rev. **39**, 1 (1975).
15. CHOPRA, I., BENNETT, P., LACEY, R. W.: J. gen. Microbiol. **343** (1973).

Address of the author:

JUDITH LANTOS
Public Health Station
H-6701 Szeged, P.O.B. 389, Hungary

CHARACTERIZATION OF ESCHERICHIA COLI SEROGROUPS CAUSING MENINGITIS, SEPSIS AND ENTERITIS

I. SEROLOGICAL PROPERTIES AND ANIMAL PATHOGENICITY OF O18, O78 AND O83 ISOLATES

ÉVA CZIRÓK, HEDDA MILCH, J. MADÁR and G. SEMJÉN

Pest County Public Health Station, Budapest, National Institute of Hygiene, Budapest and
Veterinary Research Institute, Hungarian Academy of Sciences,
Budapest

(Received July 9, 1976)

Escherichia coli O78: K80 strains isolated from an outbreak among premature and newborn infants with meningitis, sepsis and enteritis, from sporadic cases of enteritis and from healthy carriers were compared with one another and with different *E. coli* serogroups. The O78: K80 cultures uniformly failed to give the rabbit intestinal loop test and the guinea pig eye reaction and none of them contained L1 antigen. After intraperitoneal injection into mice, the organisms multiplied in the peritoneal cavity and caused bacteraemia lasting at least 2 weeks. *E. coli* strains originating from septicaemia (O78: K80, O18a,c: K?, O83: K?) showed significantly lower LD₅₀ values for mice (9×10^3 – 7×10^5) than did *E. coli* serogroups associated with infantile enteritis only (3×10^8 – 7×10^8). It is assumed that the isolates differ in pathogenicity not only from *E. coli* strains associated with “cholera-like” disease and with “dysenteriform” infection, but also from L1 antigen-containing cultures described in neonatal meningitis, and constitute a separate group characterized by an ability to cause meningitis, sepsis and enteritis within the same outbreak.

In March, 1974, in the infants ward of János Hospital, Budapest, an infant became ill with meningitis; from the cerebrospinal fluid *Escherichia coli* was cultured. Ten days later another infant acquired meningitis; the causative agent was *E. coli* similar in antibiotic resistance to the organism associated with the first case. The hospital epidemic beginning in this manner lasted for 6 months and affected 22 newborn or premature babies, 4 of whom died. At autopsy, 3 infants showed the signs of sepsis, meningitis and peritonitis; one infant had, in addition, enteric changes. Three out of the 18 surviving babies suffered from extraintestinal diseases (peritonitis, scrotal abscess), but in the rest of them enteritis predominated [1–3]. *E. coli* strains isolated from CSF, umbilical, throat or faecal specimens as well as from the environment belonged uniformly to serogroup O78 [4, 5].

In connection with the findings, three questions arose. (1) Were *E. coli* O78 strains the real causative agents? (2) Do these strains differ from other *E. coli* cultures in any feature which would explain the severe generalized form of the infection? (3) If O78 strains exhibit a peculiar pathogenicity, are there similar kinds of organisms in other *E. coli* serogroups?

To answer question 1, we examined whether the outbreak could be traced to one source, whether the isolates displayed identical features. Question 2 was attempted to be settled by serological and immunoelectrophoretic analysis and examination of enterotoxin-producing ability, persisting capacity and virulence of the strains. To elucidate question 3, the above experiments were extended to other *E. coli* serogroups associated with lethal nosocomial sepsis and meningitis.

Materials and methods

Samples. During the outbreak faeces, throat and nose swabs, CSF, wound swabs, fomites and air were examined (see Results). To collect O78 strains from other sources, in the period 1973 to 1975, 2411 faecal samples and 514 throat swabs were cultured [6]. In the course of a nursery outbreak 141 faecal samples were obtained from 47 infants with enteritis and from 28 contact infants. *E. coli* strains not belonging to serogroup O78 were isolated from the CSF and necropsy materials of 3 infants with meningitis and from the vulva of a mother.

Bacteriological examination. O and OK sera were prepared with type strains by the usual method [7] and absorbed as described earlier [8]. Colonies growing on eosin methylene blue agar were tested by slide agglutination in OK serum and transferred to agar slants. After incubation at 37 °C for 24 hr, the growth was suspended in saline, boiled for 2½ hr and tested in absorbed O serum. The strain was classified in the corresponding serogroup if agglutination was of full titre.

Antibiotic sensitivity was determined to chloramphenicol, neomycin, polymyxin B, streptomycin, tetracycline, gentamicin, kanamycin, nalidixic acid and colistin by the use of Resistest disks (Institute for Serobacteriological Production and Research Human, Budapest).

Enterotoxin was produced by two methods.

1. Whole cell lysate (WCL) was prepared by the method of GYLES and BARNUM [9] as modified by PESTI and SEMJÉN [10]. The preparation contained mainly thermolabile enterotoxin storable at 5 °C.

2. A different type of cell-free preparation was made by a slight modification of the method of SMITH and HALLS [11]. Soft agar medium was seeded with 1 ml of 18 hr broth culture and incubated at 37 °C for 24 hr. Agar was removed by filtering through linen cloth, then the culture was sonicated at 1.2 A for 30 min. After centrifugation at 10 000 rpm for 30 min the supernatant was passed through 450 µm Millipore filter. This preparation, referred to as ST+LT (thermostable + thermolabile enterotoxin), was stored at -18 °C.

Rabbit ligated loop test. New Zealand white rabbits weighing 1.8–2.6 kg were fasted 48 hr prior to operation. The animals were anaesthetized with a combination of chlorpromazine hydrochloride (Hibernal®; EGYT, Budapest; 5 mg/kg intramuscularly) and thialbarbital (Intranarcon®; Chincin, Budapest; 50 mg/kg intravenously). The jejunum was exposed through a midline incision and 9 or 10 test segments, 8 cm long, at 4 cm distances, were separated with ligatures. The first ligature was applied at a distance of 60 cm from the pylorus. Into each test segment 1 to 2 ml samples were injected. LT prepared from *E. coli* strain P99 (O141: K85a, 85b, 88a, 88b), used as control, was injected into the first and last segments. After injection of the samples the abdomen was closed. Twenty hours later the rabbits were sacrificed with an overdose of thialbarbital. The result was expressed as the ratio of the weight of fluid accumulated in the segment and the weight of the corresponding part of the intestinal wall. An index value of 1.0 or higher was regarded as a positive reaction.

Antigenic analysis of the strains was performed by the usual methods [7]. Immunoelectrophoretic examination was carried out by the technique of ØRSKOV *et al.* [12] as modified by CZIRÓK *et al.* [13].

Pathogenicity test. CFLP mice weighing 16–18 g were injected intraperitoneally with 0.5 ml of graded dilutions of 4–6 hr broth culture [14, 15]. LD₅₀ was calculated with the formula of REED and MUENCH [16]. Survival of the strains in the peritoneal cavity was estimated as described by READE and BATEMAN [17] and by WOLBERG and DEWITT [18]. The ability to cause bacteraemia was tested by the method of RAUSS and KÉTYI [19]. Bacteria were counted by streaking 0.5 ml aliquots of dilutions on 3 parallel eosin methylene blue agar plates. Strains used in these experiments are listed in Tables I and II.

Table I

Serogroup and source of E. coli strains used for mouse LD₅₀ test

Designation		Antigens	Source
			<i>Hospital outbreak</i>
	23473	O78: K80	Sepsis of newborn infant, CSF
	25049	O78: K80	Mother's milk
	25060	O78: K80	Air
	25066	O78: K80	Sepsis of newborn infant, CSF
	25076	O78: K80	Faeces of healthy nurse
	38343	O78: K80	Enteritis of newborn infant
	43805	O78: K80	Sepsis of newborn infant, umbilical swab
	45429	O78: K80	Sepsis of newborn infant, scrotal abscess
	49933	O78: K80	Faeces of infant without enteritis
	50351	O78: K80	Faeces of infant without enteritis
	58967	O78: K80	Faeces of infant without enteritis
	60112	O78: K80	Faeces of infant with enteritis
	5070	O78: K80	Faeces of infant without enteritis
Lt	321a/1	O78: K80	Faeces of infant with enteritis
EK	899/2	O78: K80	Faeces of infant without enteritis
			<i>Other sources</i>
Lsz	360a/1	O78: K80	Sporadic enteritis of infant
Lsz	395a	O78: K80	Sporadic enteritis of infant
Lsz	440f/1	O78: K80	Sporadic enteritis of infant
Lsz	444a/2	O78: K80	Sporadic enteritis of infant
Lsz	446c/1	O78: K80	Sporadic enteritis of infant
Szsz	41787	O78: K80	Enteritis of child, nursery outbreak
Szsz	43743	O78: K80	Healthy mother, faeces, nursery outbreak
Szsz	46743	O78: K80	Child convalescent from enteritis, nursery outbreak
Szsz	46722	O78: K80	Sporadic enteritis of child
Szsz	47830	O78: K80	Sporadic enteritis of child
Szsz	56588	O78: K80	Healthy adult, faeces
Szsz	64931	O78: K80	Sporadic enteritis of child
	27266	O83: K?	Vulvovaginitis of mother
	27272	O83: K?	Enteritis of infant of same mother
	17963	O18ac: K?	Sepsis of newborn infant, wound, Wernigerode
	17968	O18ac: K?	Sepsis of newborn infant, CSF, Wernigerode
	15749	O55: K59	Healthy child, faeces
	22143	O111: K58	Enteritis of infant

Table II

Serogroup, source and enterotoxigenicity of E. coli strains used for mouse bacteriaemia test

Designation	Antigens	Enterotoxin	Source
			<i>Hospital outbreak, Budapest</i>
60112	O78: K80	—	Sepsis of newborn infant
23473	O78: K80	—	Sepsis of newborn infant
25066	O78: K80	.	Sepsis of newborn infant
			<i>Other sources</i>
Lsz 446c/1	O78: K80	.	Enteritis of infant
Szsz 41787	O78: K80	—	Enteritis of infant
			<i>Outbreak, Wernigerode</i>
17963	O18a, c: K?	—	Sepsis of newborn infant
17965	O18a, c: K?	.	Sepsis of newborn infant
17968	O18a, c: K?	.	Sepsis of newborn infant
			<i>Sepsis of chicken</i>
F11+ (P307) Smith	O18a, b: K?	+	
F11- Smith	O18a, b: K?	—	
			<i>Hospital outbreak, Miskolc</i>
M 56898	O4: K?	—	Enteritis of newborn infant
M 56899	O114: K90	+	Enteritis of newborn infant
			<i>Other human source</i>
15749	O55: K59	.	Healthy child, faeces
22143	O111: K58	.	Enteritis of infant
			<i>Other animal source</i>
P 105 Söderlind	O138: K81	+(ST)	Pig

. Not examined

Results

Bacteriological examination of samples. In the course of the hospital outbreak, *E. coli* O78 was cultured from the faeces of 115 out of 280 persons (mainly infants), from the throat swabs of 35 out of 42 infants, from the nose swabs of 22 out of 27 infants, from 6 CSF samples of one infant and from 1 wound swab. Necropsy specimens (brain abscess, peritoneal exudate, lung) yielded strains from 4 infants. From the environment (air, nurses' gowns, mothers'

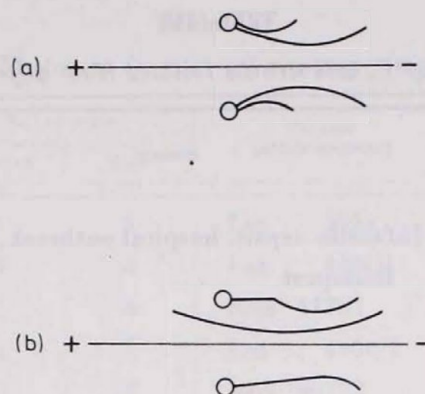


Fig. 1. Electrophoretic pattern of *E. coli* O78 strains. (a) Antiserum trough: O serum for *E. coli* O78 type strains E 38. Upper well: 100 °C 60 min antigen for *E. coli* O78 epidemic strain 23473; lower well: 60 °C 20 min antigen for strain 23473. (b) Antiserum trough: OK serum for *E. coli* O78 type strain E 38. Upper well: 100 °C 60 min antigen for strain 23473; lower well: 60 °C 20 min antigen for strain 23473

milk) 20 strains were cultured. Other enteropathogenic serogroups of *E. coli* were not detected in course of the hospital outbreak.

In the 1973–1975 survey of the incidence of *E. coli* serogroups [6], O78 strains were shown in 1.2–2% from patients with enteritis and in 1.6% from healthy persons; this serogroup was not isolated from patients hospitalized with extraintestinal diseases (tonsillitis, pneumonia, bronchitis, otitis).

Antibiotic sensitivity of *E. coli* O78. Sixty-two strains from the hospital outbreak, 21 strains collected from different parts of the country and 7 strains associated with a nursery outbreak were compared.

The epidemic strains were practically uniform in antibiotic sensitivity (resistant to chloramphenicol and, with one exception, to tetracycline). Other strains were less frequently resistant to these antibiotics.

Antigenic analysis. By cross-agglutination and cross-absorption it was shown that strain 23473 (representing isolates from the hospital outbreak), strain Szsz 41787 (nursery outbreak) and strain *E. coli* E 38 (type strain for serogroup O78: K80) contained identical O antigens. The isolates retained their K agglutinin binding capacity after heating at 100 °C for 2½ hr and contained K antigen identical with that of the type strain.

In immunoelectrophoretic pattern the isolates were also identical with the type strain and belonged to group 1Bb of the schema of ØRSKOV *et al.* [12] (Fig. 1).

Enterotoxin production. The epidemic strains produced neither LT nor ST.

Mouse pathogenicity. At first 4 epidemic strains and 4 cultures isolated from other sources were examined for LD₅₀. Groups of 5 mice were injected with graded doses ranging from 5×10^8 to 5×10^2 cells. Results of these experiments were read 1 day after injection (see the corresponding column of Table

Table III
Mouse LD₅₀ of E. coli strains isolated from different sources

Designation	O antigen	Source	LD ₅₀	
			1 day	6 days
60112	78	Infantile sepsis, hospital outbreak, Budapest	2×10^6	7×10^5
5070			3×10^6	6×10^5
25049			3×10^6	7×10^5
23473			2×10^6	4×10^5
Szsz 41787	78	Enteritis of children, sporadic cases and nurseries	1×10^7	7×10^6
Lsz 446c/1			3×10^7	7×10^6
Szsz 46722			6×10^7	6×10^6
Lsz 360a/1			3×10^7	10^6
17963	18a, c	Sepsis of newborn infant, Wernigerode outbreak	3×10^7	9×10^3
17968			5×10^7	4×10^4
27272	83	Sepsis of infant, sporadic case	4×10^7	3×10^4
27266			1×10^7	3×10^4
15749	55	Faeces of healthy child	3×10^8	7×10^8
22143	111	Enteritis of infant	4×10^8	3×10^8

III). The epidemic strains showed higher pathogenicity than the other O78 strains. The difference was even more marked at the 6-day reading.

In subsequent tests, groups of 5 mice were injected intraperitoneally with 10^6 cells of 15 epidemic and 12 other O78 strains (Table IV).

The results of the preliminary tests were confirmed: the difference in pathogenicity between epidemic and other strains was significant by the χ^2 test ($p < 0.001$).

To examine the survival of the isolates, groups of 22 mice were injected intraperitoneally with 10^6 cells and survival of the bacteria was tested at 2, 17, 24 and 72 hr intervals (Table V). In the first two hours the number of bacteria remained the same as in the inoculum with all strains. Later the epidemic strain multiplied in the peritoneal cavity, whereas other strains showed decreasing counts.

Recovery of different *E. coli* strains from the blood of intraperitoneally injected mice supported these findings. Figure 2 shows the results of experiments with the strains listed in Table II. All strains were recovered from the blood samples taken 2 hr after injection. The blood count for *E. coli* O78 increased after 17 hr and remained at the 10^7 /ml level for at least 13 days. In contrast,

Table IV

Lethal effect of E. coli O78 strains in mice after intraperitoneal injection of 10⁶ cells

Strains hospital outbreak Budapest	No. of mice		Strains (other sources)	No. of mice	
	injected	died		injected	died
60112	5	5	Lsz 395a	5	2
5070	5	4	Lsz 440f/1	5	0
25049	5	4	Szsz 41787	5	2
23473	5	5	Lsz 446c/1	5	1
45429	5	3	Szsz 46722	5	4
Lt 321a/1	5	5	Lsz 360a/1	5	5
Ek 899/2	5	3	Szsz 43743	5	0
25076	5	2	Lsz 444a/2	5	0
38343	5	4	Szsz 56588/2	5	0
25060	5	5	Szsz 47830	5	1
43805	5	4	Szsz 64931	5	0
58967	5	4	Szsz 46743	5	0
25066	5	0			
50351	5	0			
49933/1	5	2			
	75	50		60	15

Table V

Time course of intraperitoneal bacterial counts for E. coli O78 strains injected into mice

Source	Hours after injection	Bacterial counts recovered						
		<10 ²	10 ² -10 ³	10 ³ -10 ⁴	10 ⁴ -10 ⁵	10 ⁵ -10 ⁶	10 ⁶ -10 ⁷	10 ⁷ -10 ⁸
Out- break	2						○ ○ ○ ● ● ●	
	17				● ● ● ●	○		○ ○
	24					○ ●		○ ○ ●
	72					● ●	○ ○ ○ ●	
Other	2						□ ■	
	17				□ ■	□ ■		
	24			■	■	□	□ ■	
	72	■	□ ■ ■		□ □			

○ strain 60112

● strain 23473

□ strain Szsz 41787

■ strain Lsz 446c/1

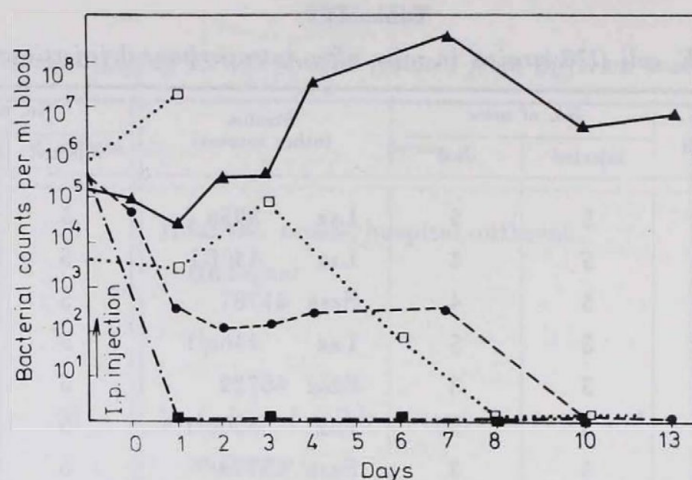


Fig. 2. Time course of recovery of different *E. coli* strains from the blood of mice injected intraperitoneally. $\blacktriangle$ — $\blacktriangle$ Epidemic O78 strains; $\square$ $\square$ epidemic O18 strains; $\blacksquare$ — $\blacksquare$ other O18 strains; $\bullet$ — $\bullet$ serogroup O4, O55, O111, O114 and O138 strains

other strains, irrespective of their source and enterotoxigenicity, showed a decrease in number and had disappeared from the blood by the 10th day after injection (Tables VI and VII).

The guinea pig eye test [20] was negative for all strains.

In addition to O78, two serogroups of *E. coli* were isolated from septic conditions. *E. coli* O18a, c strains were isolated at the Department of Paediatrics,

Table VI

Time course of blood counts for *E. coli* O78 strains after intraperitoneal injection of mice

Time after injection	Strains, blood counts					Average blood counts**
	60112	23473	Szsz 41787	Lsz 446c/1	25066	
0 (injected bacteria)	1.4×10^5	1.0×10^4	1.3×10^5	4.1×10^5	3.9×10^5	$(2.2 \pm 0.78) \times 10^5$
2 hr	$1.1 \times 10^{4*}$	9.9×10^4	0	.	.	$(4.1 \pm 2.5) \times 10^4$
17 hr	8.3×10^2	4.3×10^5	3.3×10^4	.	.	$(1.5 \pm 1.1) \times 10^5$
24 hr	6.0×10^2	2.8×10^4	3.3×10^4	5.6×10^4	3.0×10^4	$(2.06 \pm 1.0) \times 10^4$
2 days	3.3×10^5	3.6×10^5	3.4×10^5	.	.	$(2.42 \pm 1.4) \times 10^5$
3 days	3.3×10^4	5.7×10^3	5.0×10^4	1.2×10^6	3.0×10^4	$(2.2 \pm 1.9) \times 10^5$
4 days	9.3×10^6	2.2×10^6	9.5×10^7	.	.	$(2.8 \pm 2.3) \times 10^7$
7 days	1.3×10^8	4.4×10^6	.	.	.	$(4.9 \pm 4.3) \times 10^7$
10 days	4.6×10^6	3.4×10^5	.	0	0	$(1.62 \pm 1.1) \times 10^6$

* Average of 3 experiments

** Average of all experiments

0 Bacteria not recovered

. Not examined

Table VII

Time course of blood counts for *E. coli* strains other than O78 after intraperitoneal injection into mice

Time after injection	Strains, blood counts					Average blood counts**
	O138 P 105	O55 15749	O111 22143	O4 M 56898	O114 M 56899	
0 (injected bacteria)	4.3×10^5	3.3×10^5	2.8×10^5	3.2×10^5	3.9×10^5	$(3.5 \pm 0.3) \times 10^5$
2 hr	$2.9 \times 10^{4*}$	9.9×10^2	1.3×10^5	.	.	$(5.15 \pm 2.52) \times 10^4$
17 hr	2.8×10^2	4.0×10^2	1.8×10^3	.	.	$(8.29 \pm 3.91) \times 10^2$
24 hr	5.4×10^1	4.0×10^1	5.3×10^1	0	8.4×10^2	$(1.63 \pm 1.18) \times 10^2$
2 days	0	5.4×10^1	6.0×10^1	.	.	$(3.78 \pm 2.32) \times 10^1$
3 days	0	2.6×10^2	1.4×10^2	0	0	$(6.0 \pm 3.4) \times 10^1$
4 days	0	1.3×10^1	3.4×10^2	.	.	$(1.2 \pm 1.1) \times 10^2$
7 days	6.0×10^1	0	0	.	.	$(2.0 \pm 0.96) \times 10^1$

* Average of 3 experiments

** Average of all experiments

0 Bacteria not recovered

. Not examined

Wernigerode, GDR, where they caused an outbreak of meningitis and sepsis in infants and enteritis in children above one year of age. *E. coli* O83 was cultured from the vulva of a mother (27266) and from the CSF of her infant (27272) who had probably been infected during delivery and died from pneumonia and meningitis 3 days later.

These O18a, c and O83 strains and the O78 strains showed practically no difference in LD₅₀ (Table III). Strains isolated from healthy persons and from patients with enteritis were less pathogenic: their LD₅₀ was markedly higher than that for septic strains.

As to the ability to invade the blood stream of mice, *E. coli* O18a, c strains associated with meningitis were fairly similar to O78 cultures. As seen in Fig. 2, O18 counts in blood also showed an increase for 3 days; the difference in the further course of O78 and of O18 bacteraemia may have been due to the lower initial dose of O18 (the organism killed the animals if it was applied in the same dose as were the O78 strains). The epidemic O18 strains were compared with Smith strains of *E. coli* O18 F11⁻ (isolated from chicken sepsis) and F11⁺ (enterotoxin-producing derivative of the former). The two Smith strains behaved uniformly and, unlike septic O18 strains, they failed to multiply in the blood of mice.

Discussion

Our paper characterizes certain *E. coli* serogroups in association with pathogenic properties not described in the literature. In humans, serogroup O78 has been known to cause only infantile diarrhoea [21–23]. In newborn calves and lambs, however, this serogroup proved responsible for septicaemia [24–27].

The assumption that *E. coli* O78 isolates were responsible for the hospital outbreak of meningitis, sepsis and enteritis, was supported by the fact that the strains were uniform in antibiotic resistance and that O78 strains occurred infrequently in healthy individuals.

The second question was whether epidemic O78 strains differed in animal pathogenicity from other O78 strains. First we examined the isolates for the presence of antigen K1(L) which, according to ROBBINS *et al.* [28], occurs in 86% of *E. coli* strains cultured from purulent meningitis of infants but only in 11–13% of strains from patients without meningitis. None of our O78 isolates contained L-type antigen; they were all characterized by a B-type K antigen identical with that of the standard strain.

Our finding that O78 strains failed to produce enterotoxin disagreed with literary data classifying these cultures into the “choleraform” group of *E. coli*. (Our results were kindly checked and confirmed by Professor I. KÉTYI and his associates, Institute of Microbiology, University Medical School, Pécs, by Craig’s intradermal rabbit test and by the oral test in suckling mice). In contrast, ETKIN and GORBACH [29], SACK *et al.* [30] and EVANS *et al.* [31] described enterotoxigenic O78 strains. SOJKA [26] regarded O78 strains as belonging to the group associated with “travellers’ diarrhoea”.

Our epidemic O78 strains differed from other O78 strains in being able to multiply in the peritoneal cavity of the mouse. From enterotoxin-producing and animal pathogenic *E. coli* strains they differed in their capability of maintaining a prolonged bacteraemia.

E. coli O18a, c strains isolated from an outbreak of sepsis and enteritis and *E. coli* O78 strains associated with sepsis were similar in animal pathogenicity to epidemic O78 strains. This finding is in agreement with that of JENKIN and ROWLEY [32], who showed prolonged bacteraemia for virulent bacteria. Our mice injected with virulent organisms developed endotoxin shock with pyrexia, ruffled fur, conjunctival exudation and diarrhoea. The same symptoms were described by WOLBERG and DEWITT for virulent strains [18]. According to MEDEARIS and KENNY [15], in certain *E. coli* strains virulence is associated with the ability to multiply in the peritoneal cavity. As the present investigations confirmed that peritoneal multiplication and lethality were correlated, it may be assumed that the mouse LD₅₀ test is an important diagnostic criterion for *E. coli* associated with enteric + septic infections.

Results presented in this paper are in agreement with the finding of WOLBERG [18], who examined the progression of intraperitoneal infection and showed that the bacteria were always less in number in the circulation than in the peritoneal cavity.

Our examinations were already in progress when SARFF *et al.* [33] and ØRSKOV and ØRSKOV [34] published papers on the isolation of *E. coli* O18, O78 and O83 from cerebrospinal fluid and blood, but made no reference as to whether the patients had or had not enteric symptoms.

In conclusion, it may be assumed that *E. coli* strains examined in this work are able to cause sepsis and meningitis not only because they invade immunologically immature infants or superinfect patients damaged by other diseases [35], but also because they have an increased pathogenicity and virulence.

As *E. coli* O18, O78 and O83 strains are capable of causing sepsis, meningitis and enteritis within the same outbreak, they ought to be classified into a group separate from *E. coli* strains associated with infantile diarrhoea, cholera-like enteritis ("travellers' diarrhoea") or dysenteriform disease.

Acknowledgement. We are indebted to Miss GABRIELLA PUTNOKI for skilled technical assistance.

REFERENCES

1. SERE, G., KENDE, É., ADAMIS, É., BÉKÉSY, Zs., CZIRÓK, É., JÁKÓ, Z., KORMOS, E., KUBINYI, J., MAXIMOVA, G., MIHÁLYFI, I.: Meeting of the Hungarian Society for Infectious Diseases. March 23, 1975, Budapest.
2. SZABÓ, L., VASS, M., PINTÉR, G., FORRAI, M.: Meeting of the Hungarian Society for Infectious Diseases. March 23, 1975, Budapest.
3. FARKAS, É., HAJDI, Gy., GERŐ, A., SOÓS, M., BENE, M.: Meeting of the Hungarian Society for Infectious Diseases. March 23, 1975, Budapest.
4. KENDE, É., SERE, G., CZIRÓK, É., BÉKÉSY, Zs., JÁKÓ, Z., KORMOS, E., KUBINYI, J., MAXIMOVA, G., MIHÁLYFI, I., NYOMÁRKAI, I., NÉMEDI, L., SALFAI, G., ADAMIS, É.: *Egészségtudomány* **19**, 79 (1975).
5. CZIRÓK, É., MILCH, H., MADÁR, J., DOMBI, I., ADAMIS, É.: *Egészségtudomány* **19**, 85 (1975).
6. CZIRÓK, É., MADÁR, J., BUDAI, J., STVERTECZKY, Zs., KERTÉSZ, A., DOMBI, I., GYENGÉSI, L.: *Acta microbiol. Acad. Sci. hung.* **23**, 359 (1976).
7. EDWARDS, P. R., EWING, W. H.: *Identification of Enterobacteriaceae*, 2nd ed. Burgess, Minneapolis 1962.
8. CZIRÓK, É., MADÁR, J., MILCH, H., GYENGÉSI, L.: *Egészségtudomány* **17**, 275 (1973).
9. GYLES, C. L., BARNUM, D. A.: *J. infect. Dis.* **120**, 419 (1969).
10. PESTI, L., SEMJÉN, G.: *Acta vet. Acad. Sci. hung.* **23**, 227 (1973).
11. SMITH, H. W., HALLS, S.: *J. Path. Bact.* **93**, 531 (1967).
12. ØRSKOV, F., ØRSKOV, I., JANN, B., JANN, K.: *Acta path. microbiol. scand. B.* **79**, 142 (1971).
13. CZIRÓK, É., MILCH, H., STVERTECZKY, Zs., HÉRMÁN, G., LOSONCZY, G.: *Acta microbiol. Acad. Sci. hung.* **22**, 299 (1975).
14. SMITH, H. W.: *J. gen. Microbiol.* **83**, 95 (1974).
15. MEDEARIS, D. N. JR., KENNY, J. F.: *J. Immunol.* **101**, 534 (1968).
16. REED, L. J., MUENCH, H.: *Amer. J. Hyg.* **27**, 493 (1938).
17. READE, P. C., BATEMAN, R.: *J. infect. Dis.* **120**, 471 (1969).
18. WOLBERG, G., DEWITT, C. W.: *J. Bact.* **100**, 730 (1969).

19. RAUSS, K., KÉTYI, I.: Z. Immunität.-Forsch. **123**, 173 (1962).
20. SERÉNY, B.: Acta microbiol. Acad. Sci. hung. **4**, 367 (1957).
21. EWING, W. H., DAVIS, B. R., MONTAGUE, T. S.: Studies on the Occurrence of *Escherichia coli* Serotypes Associated with Diarrhoeal Disease. C.D.C. Atlanta, Georgia 1963.
22. ØRSKOV, F.: Acta path. microbiol. scand. **31**, 51 (1952).
23. KAUFFMANN, F.: Classification of Bacteria. Munksgaard, Copenhagen 1975. p. 148.
24. WRAMBY, G.: Investigations into the Antigenic Structure of *Bact. coli* Isolated from Calves. Appelbergs, Uppsala 1948. cit. in 21.
25. GLANTZ, P. J.: Ann. N. Y. Acad. Sci. **176**, 67 (1971).
26. SOJKA, W. J.: Vet. Bull. **41**, 509 (1971).
27. MANZ, J.: Über die serologische Differenzierung von *Escherichia coli* Stämmen vom Kalb. Inaug. Diss. München 1971.
28. ROBBINS, J. B., MCCracken, G. H. JR., GOTSCHICH, E. C., ØRSKOV, F., ØRSKOV, I., HANSON, L. A.: New Engl. J. Med. **290**, 1216 (1974).
29. ETKIN, S., GORBACH, S. L.: J. Lab. clin. Med. **78**, 81 (1971).
30. SACK, R. B., GORBACH, S. L., BANWELL, J. G., JACOBS, B., CHATTERJEE, B. D., MITRA, R. C.: J. infect. Dis. **123**, 378 (1971).
31. EVANS, D. J., EVANS, D. G. JR., GORBACH, S. L.: Infect. Immun. **8**, 725 (1973).
32. JENKIN, C. R., ROWLEY, D.: J. exp. Med. **114**, 363 (1961).
33. SARFF, L. D., MCCracken, G. H. JR., SCHIFFER, M. S., GLODE, M. P., ROBBINS, J. B., ØRSKOV, I., ØRSKOV, F.: Lancet **1**, 1099 (1975).
34. ØRSKOV, F., ØRSKOV, I.: Acta path. microbiol. scand. B. **83**, 595 (1975).
35. COCCHI, P., BARTOLOZZI, G., TAYLOR, J., BETTELHEIM, K. A.: Helv. Paediat. Acta **22**, 313 (1967).

Address of the authors:

ÉVA CZIRÓK, JÁNOS MADÁR
Pest County Public Health Station
H-1428 Budapest P.O.B. 55, Hungary

HEDDA MILCH
National Institute of Hygiene
H-1966 Budapest P.O.B. 64, Hungary

GÁBOR SEMJÉN
Veterinary Research Institute, Hungarian Academy of Sciences
H-1581 Budapest P.O.B. 18, Hungary

CHARACTERIZATION OF *ESCHERICHIA COLI* SEROGROUPS CAUSING MENINGITIS, SEPSIS AND ENTERITIS

II. CLASSIFICATION OF *ESCHERICHIA COLI* O78 STRAINS BY PHAGE SENSITIVITY, COLICIN TYPE AND ANTIBIOTIC RESISTANCE

HEDDA MILCH, ÉVA CZIRÓK, J. MADÁR and G. SEMJÉN

National Institute of Hygiene, Budapest,
Pest County Public Health Station, Budapest and
Veterinary Research Institute, Hungarian Academy
of Sciences, Budapest

(Received July 9, 1976)

Escherichia coli O78: K80 strains isolated from an outbreak of meningitis, sepsis and enteritis in infants, were compared with O78: K80 strains from sporadic cases of enteritis, healthy carriers and animals. The strains were uniform in antigenic structure and phage pattern but differed in colicinogenicity. The epidemic strains and calf-pathogenic cultures produced colicin V, the remaining isolates were characterized by other types of colicin or were not colicinogenic. Col V⁺ strains multiplied in the mouse peritoneal cavity more readily and killed the animals at significantly lower doses than did col V⁻ strains. One half of antibiotic resistant O78: K80 strains carried R factor. The spread of R factor could be followed by phage restriction experiments.

An outbreak of meningitis and enteritis in a newborn infants ward was shown to be associated with *Escherichia coli* O78: K80 [1, 2]. The strains were compared with other *E. coli* O78: K80 cultures isolated from different sources. Phage typing [3], colicin typing and lysogenicity tests were supplemented with (i) determination of the type of colicin, (ii) col factor transfer experiments, (iii) antibiotic resistance spectrum, (iv) detection of R factor in antibiotic resistant strains, (v) characterization of the R factor.

Materials and methods

Bacterial cultures. Of 177 human strains 2 were isolated from CSF, 131 from faeces, 18 from throat, 5 from nose, 3 from other samples. Eighteen strains were collected from the environment (air, fomites). The 75 epidemic strains originated from János Hospital, 55 from László Hospital, 12 from other hospitals, 8 from sporadic non-hospitalized cases of enteritis and 27 from nurseries in Budapest. Four out of the 6 animal strains were isolated from fibrinous inflammation of the air sac of turkeys, 2 from toxæmic calves' small intestine and mesenteric lymph node. The calves' strains were isolated and kindly supplied by Dr. J. VARGA, Institute of Epidemiology, University Veterinary School, Budapest.

Serological examinations were performed as described previously [1].

Phage pattern of the isolates was determined by the method of MILCH and GYENES [3] using 30 *E. coli* typing phages. Phage 4 of the original set was adapted to an O78: K80 strain isolated from the outbreak (phage 4a) and to an O78: K80 strain culture from mild enteritis in a nursery (phage 4b).

Colicinogenicity and lysogenicity were examined as described by MILCH and GYENES [3].

Colicin type was determined by the method of LEWIS [4] with FRÉDERICQ's indicator and colicin producing strains [5].

Colicin sensitivity was tested with FRÉDERICQ's colicin producing strains [5].

Antibiotic sensitivity was examined with Resistest disks (Institute for Serobacteriological Production and Research Human, Budapest) to chloramphenicol, ampicillin, streptomycin, tetracycline, neomycin, kanamycin, colistin, polymyxin B, nalidixic acid and gentamicin.

Detection of R and col plasmids with conjugation. The donor strain was cultured in broth at 37 °C for 18 hr, then a 0.5 ml portion was transferred into 4.5 ml broth. After incubation at 37 °C for 2 hr, a 0.5 ml portion of the subculture was again transferred into 4.5 ml broth and 1.0 ml of the recipient culture, which had been grown in the same manner, was added. The mixed culture was incubated at 37 °C for 5 hr then streaked on agar plates containing antibiotics. For the detection of col plasmid, 200 colonies of each culture were examined by the overlayer technique using *E. coli* K12.

Recipient strains. *E. coli* Hfr lac⁻ Nal^r was used for detecting R factor. Col factor was demonstrated with the above strain and with *E. coli* PA 309 Sm^r L⁻ F⁻.

Fi character was determined by MS₂ and f₂ male specific RNA phages decreasing or eliminating lysis of *E. coli* Hfr strain.

Phage restriction experiments were performed with phages λ, T1–T7, P1 and with the 30 phages of the *E. coli* typing set [3].

E. coli Hfr strain was examined after R-factor incorporation by the spot test. The recipient Hfr strain was previously examined at RTD of typing phages. If the phage lysing the Hfr strain failed to act at the same dilution on the R factor-carrying strain or showed a decreased activity against it, the efficiency of plating (e.o.p.) was determined. The degree of restriction was estimated from the relative e.o.p. values.

Culture medium. After conjugation the cultures were streaked on eosin-methylene blue medium containing antibiotics at inhibitory concentrations: nalidixic acid (50 µg/ml), tetracycline (50 µg/ml), ampicillin (50 µg/ml), chloramphenicol (25 µg/ml), kanamycin (20 µg/ml) and streptomycin (50 µg/ml).

Ligated rabbit intestinal loop test was carried out as described by CZIRÓK *et al.* [1] using enterotoxin prepared with the methods of GYLES and BARNUM [6], PESTI and SEMJÉN [7] and SMITH and HALLS [8].

Mouse pathogenicity was examined by intraperitoneal injection of CFLP mice weighing 16–18 g [9]. Survival of the bacteria in the peritoneal cavity of mice was tested as described previously [1].

Results

Phage type distribution of isolates is shown in Fig. 1. Out of the 183 *E. coli* O78: K80 strains 75 were cultured in the course of the János Hospital outbreak. These strains were fairly uniform in phage pattern: 65 were characterized by (4), 4a, 4b and the rest by 4a, 4b (the figure in brackets indicates weak lysis). Fifty-four out of 55 strains from the László Hospital belonged to the above phage patterns, one strain to 2, 3, 4, 4a, 4b, 12. Figure 1 shows that the majority of strains isolated from other sources were also of phage patterns (4), 4a, 4b or 4a, 4b. Exceptions were one 4, 4a, 4b, 12 and one 4, 4a, 4b, 22 from other hospitals, one 2, 3, 4, 4a, 4b, 12 and one untypable from nurseries.

On the basis of phage pattern there was no difference between human and animal strains or between cultures originating from patients and healthy carriers. The purpose of subsequent experiments was, therefore, to find a property distinguishing epidemic strains from other ones.

Colicinogenicity. Of 183 *E. coli* O78 strains 122 (66.6%) were colicinogenic (col⁺). The distribution of col⁺ and col⁻ strains according to source is shown in Table I. Colicinogenic strains predominated in the outbreak and in animals, were isolated fairly frequently from László Hospital, but occurred significantly less often in nurseries and sporadic cases.

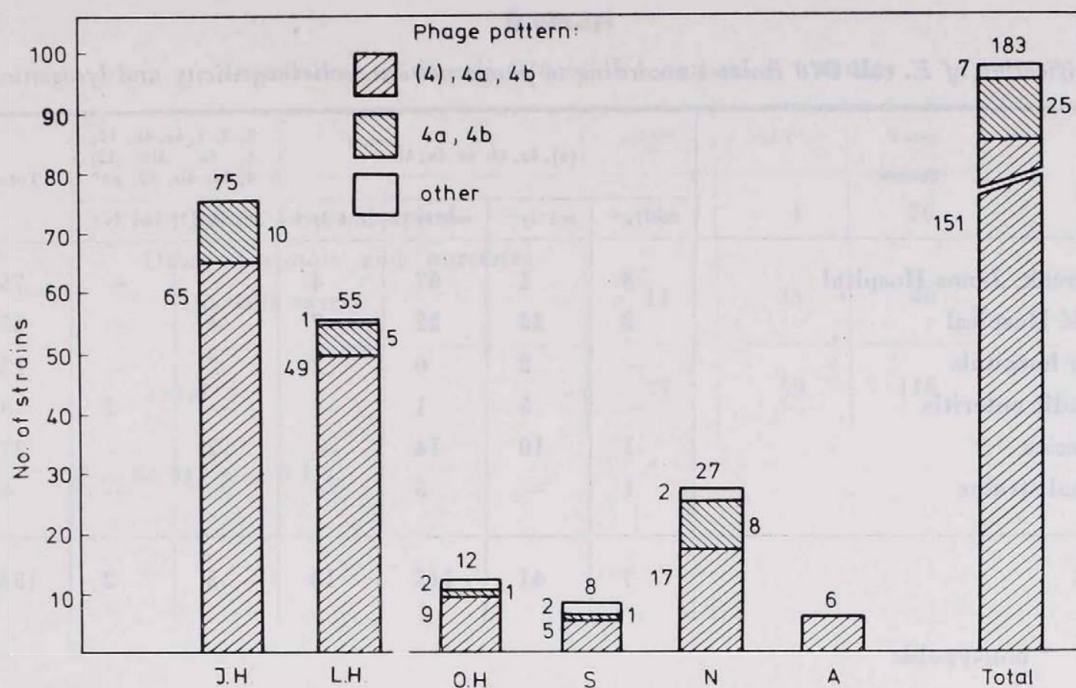


Fig. 1. Distribution of *E. coli* O78 strains according to phage pattern. J. H. = János Hospital; L. H. = László Hospital; O. H. = other hospitals; S = sporadic cases of enteritis; N = nurseries; A = animal strains

Lysogenicity. Only 15 out of the 183 strains were lysogenic (8.2%). These cultures were distributed rather evenly: János Hospital, 4; László Hospital, 7; other hospitals, 2; sporadic cases, 2 strains. Lysogenic cultures were not encountered in nurseries.

Classification of *E. coli* O78 strains by phage pattern, colicinogenicity and lysogenicity is shown in Table II. The strains fell into 6 groups, or into 9 groups if rare phage patterns were also considered.

Table I

Distribution of E. coli O78 col⁺ and col⁻ strains according to source

Source	col ⁺	col ⁻	Total
Outbreak, János Hospital	70	5	75
László Hospital	24	31	55
Other hospitals	6	6	12
Sporadic enteritis	1	7	8
Nurseries	15	12	27
Animals (calf, turkey)	6	—	6
Total	122	61	183

Table II

Classification of *E. coli* O78 isolates according to phage pattern, colicinogenicity and lysogenicity

Source	(4), 4a, 4b or 4a, 4b				2, 3, 4, 4a, 4b, 12; 4, 4a, 4b, 12; 4, 4a, 4b, 22; nt*		Total
	col+ly+	col-ly-	col+ly+	col-ly-	col-ly-	col-ly+	
Outbreak, János Hospital	3	1	67	4	—	—	75
László Hospital	2	23	22	7	1	—	55
Other hospitals	—	2	6	2	2	—	12
Sporadic enteritis	—	5	1	—	—	2	8
Nurseries	1	10	14	—	2	—	27
Animal strains	1	—	5	—	—	—	6
Total	7	41	115	13	5	2	183

* nontypable

Colicin type distribution of the colicinogenic *E. coli* O78 strains is shown in Table III and Fig. 2. Col V⁺ strains were predominant in the outbreak and occurred also in the László Hospital, but probably as a result of an epidemiological connection with the outbreak in János Hospital. The two col V⁺ strains isolated from symptomless infants in a nursery could also be traced to the

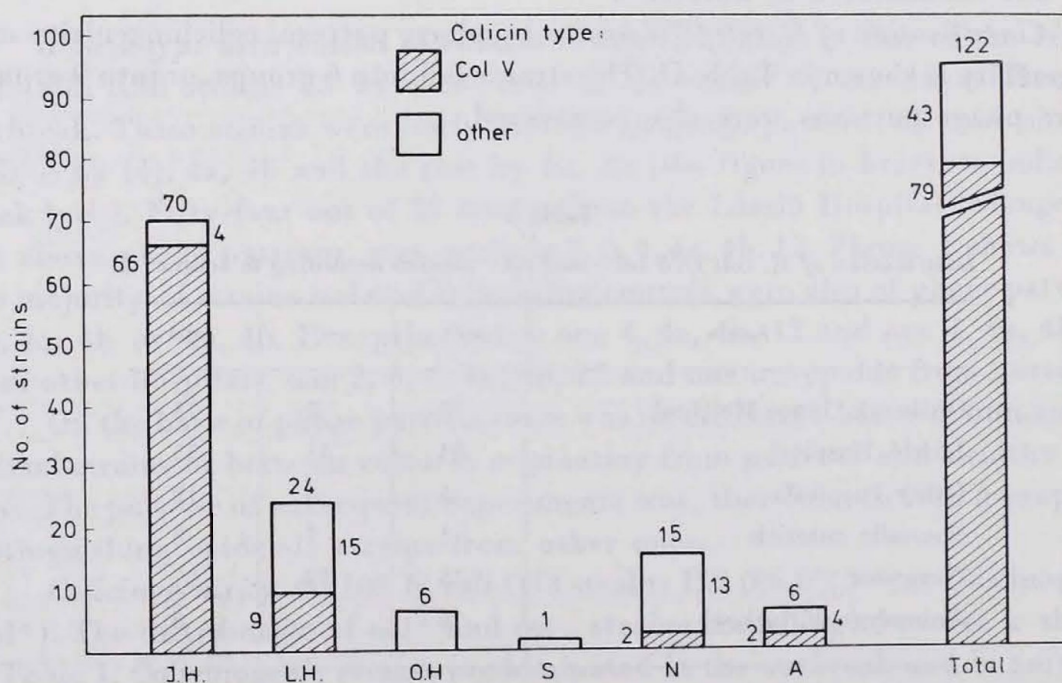


Fig. 2. Distribution of *E. coli* O78 strains according to colicin type. For abbreviations, see Fig. 1

Table III
Distribution of E. coli O78 col V⁺ and col V⁻ strains

Source	col V ⁺	col V ⁻	Total
Outbreak, János Hospital	66	4	70
Other hospitals and nurseries (sporadic cases)	11	35	46
Total	77	39	116

$$\chi^2 = 64.91, p < 0.1\%$$

same origin: both infants were born in János Hospital at the time of the outbreak. Two V⁺ cultures out of the 6 colicinogenic animal strains were isolated from toxæmic calves; the 4 colicinogenic cultures from turkeys produced different colicins (type E₂ and unclassified).

V colicin transfer. The 66 V⁺ strain was crossed with *E. coli* Hfr and PA F⁻ strains. Col V property was not transferable in these experiments.

Antibiotic sensitivity. Table IV presents the antibiotic resistance spectrum of O78 strains from various sources. The majority of isolates from the outbreak showed identical resistance pattern (chloramphenicol, tetracycline and ampicillin).

Part of the strains isolated from the László Hospital and from nurseries were sensitive to antibiotics and the rest showed different resistance patterns. Among animal strains, those isolated from calves were resistant to tetracycline + kanamycin or to chloramphenicol + kanamycin, whereas turkey strains were resistant to streptomycin + tetracycline.

Demonstration of R factor. Eighty (74 human and 6 animal) antibiotic resistant strains were tested for R factor (Table V). More than one half of cultures from János Hospital carried R factor; in strains from other hospitals the presence of R factor was less frequent. All three nursery strains contained R factor. Although the transferability of col V plasmid was not shown, it could presumably be transferred with the R factor *in vivo*. Accordingly, we examined whether the presence of R factor was associated with col V character. Results are shown in Table VI. The incidence of R⁺ strains among col V⁺ and col V⁻ cultures differed significantly ($\chi^2 = 4.426$; $p < 5\%$), as also the incidence of Rfi⁺ strains among col V⁺ and col V⁻ strains ($\chi^2 = 6.825$; $p < 1\%$).

Table IV
Antibiotic sensitivity pattern

Source	No. of sensitive strains	No. of strains resistant					
		C	T	A	CT	CK	SC
Outbreak, János Hospital	9	—	—	—	6	—	—
László Hospital	22	7	2	1	2	—	1
Other hospitals	3	—	3	—	—	—	—
Sporadic enteritis	2	—	4	—	2	—	—
Nurseries	8	1	5	—	—	—	—
Animal strains	—	—	—	—	—	1	—
Total	44	8	14	1	10	1	1

Antibiotics: C = chloramphenicol, T = tetracycline, S = streptomycin, A = ampicil-

Classification of R factor by f_i character. R factors can primarily be classified on the basis of f_i character (conjugation inhibition). Thirty out of the 39 R factors were f_i^+ . Out of the R factors of 30 col V⁺ strains 26 were f_i^+ . Two human col V⁺ strains originating from János Hospital carried f_i^- R factor; these strains had been cultured from lethal brain abscess and meningitis.

Characterization of R factor by phage restriction. Inhibition of phage multiplication by R factor was expressed as the average of relative e.o.p. values. Table VII shows phage restriction by f_i^+ R factors different in origin. R factors of the 26 strains isolated from the outbreak, the László Hospital, and the nursery restricted the same phages, although they were different in antibiotic

Table V
Presence of R factor in antibiotic resistant E. coli O78 strains

Source	No. of strains	No. of R ⁺ strains	No. of R ⁻ strains
Outbreak, János Hospital	50	27	23
Other hospitals	21	7	14
Nurseries	3	3	—
Animals	6	2	4
Total	80	39	41

of *E. coli* O78 strains

to antibiotics								Total
ST	TA	TK	CTA	SCA	SCT	SCTA	SCTNCo	
1	—	—	52	—	—	7	—	75
—	2	—	6	3	5	4	—	55
—	2	—	4	—	—	—	—	12
—	—	—	—	—	—	—	—	8
—	—	5	—	—	4	2	2	27
4	—	1	—	—	—	—	—	6
5	4	6	62	3	9	13	2	183

lin, K = kanamycin, N = neomycin, Co = colistin

resistance spectrum. Chloramphenicol, tetracycline and ampicillin resistance present in most hospital isolates could be transferred to the sensitive recipient strain. Four strains from János Hospital and László Hospital differed in R factor restriction as compared to other isolates. All human *fi*⁺ R factors were restrictive whereas *fi*⁻ R factors were not. The *fi*⁻ R factor of two O78 strains isolated from the intestine and lymph nodes of calves died from toxæmia

Table VI

Distribution of *E. coli* O78 strains according to R⁺, R⁻, R_{fi} character and colicinogenicity

Characteristics	No. of strains
R ⁺ <i>fi</i> ⁺ col V ⁺	26
R ⁺ <i>fi</i> ⁻ col V ⁺	4
R ⁻ col V ⁺	23
R ⁺ <i>fi</i> ⁺ col Ia ⁺ ?	1
R ⁺ <i>fi</i> ⁻ col E ₂ ⁺ ?	2
R ⁻ col E ₂ ⁺ ?; col Ia ⁺ ?	11
R ⁺ <i>fi</i> ⁺ col ⁻	3
R ⁺ <i>fi</i> ⁻ col ⁻	3
R ⁻ col ⁻	8
Total	81

$$\chi^2 = 6.825 \quad p < 1\%$$

Table VII
Phage restriction by fi^+ R factors
Total number

Source	Antibiotic resistance pattern	Resistance determinants transferred	Phage	
			λ	T_1
Outbreak (János Hospital)	CTA	CTA		
László Hospital, nurseries	CTAS	CTA	10^{-1}	0.9×10^{-2}
	CA	A		
	CTA	A		
Outbreak (János Hospital)	CTA	CTA	0.4×10^{-1}	0.8×10^{-2}
	CTAS	CTA		
Outbreak (János Hospital)	CTA	CTA	1.4×10^{-1}	0.3×10^{-1}

Table VIII

Pathogenicity of E. coli O78 col V⁺ and col V⁻ strains in mice after intraperitoneal injection

col V ⁺ strains			col V ⁻ strains		
Designation	No. of mice		Designation	No. of mice	
	injected	died in 8 days		injected	died in 8 days
60112	5	5	Lsz 360a/1	5	5
23473	5	5	Szsz 46722	5	4
Lt 321a/1	5	5	49933/1	5	2
25060	5	5	Szsz 41787	5	2
5070	5	4	Szsz 47830	5	1
25049	5	4	Lsz 446c/1	5	1
38343	5	4	Szsz 56588/2	5	0
43805	5	4	25066	5	0
58967	5	4	Szsz 64931	5	0
45429	5	3	Szsz 43743	5	0
Ek 899/2	5	3	Lsz 444a/2	5	0
25076/1	5	2	50351	5	0
Lsz 395/a	5	2	Szsz 46743	5	0
Lsz 440f/1	5	0			
Total	70	50	Total	65	15

$$\chi^2 = 31.6, \quad p < 0.1\%$$

of *E. coli* O78 strains
of *fi*⁺ R strains, 30

restriction						No. of strains
T ₃	T ₄	T ₅	T ₆	23	24	
1.5 × 10 ⁻²	1.5 × 10 ⁻²	2.6 × 10 ⁻²	2.6 × 10 ⁻¹	0.8 × 10 ⁻³	1	15
						9
						1
						1
4.5 × 10 ⁻³	1.5 × 10 ⁻²	2 × 10 ⁻²	1	0.8 × 10 ⁻³	1	1
						1
0.6 × 10 ⁻²	1.5 × 10 ⁻²	1.6 × 10 ⁻²	7.5 × 10 ⁻¹	0.3 × 10 ⁻⁸	1.4 × 10 ⁻²	2

differed in this respect: one caused no restriction the other restricted phages T2 and 4, 6, 16, 23, 24. This restriction pattern differed from patterns for human *fi*⁺ R factors.

Enterotoxin production. Four col V⁺ strains from the outbreak and 2 col⁻ nursery strains were tested. As compared to the standard enterotoxin-producing strain, they were negative.

Mouse pathogenicity. Intraperitoneal injection of mice with *E. coli* O78 strains [1] showed that col V⁺ strains were lethal to a significantly greater number of animals than col V⁻ cultures (Table VIII).

Table IX

Survival of E. coli O78 col V⁺ and col V⁻ strains in the peritoneal cavity of mice

Strain	Average no. of cells recovered				
	injected	2 hr	17 hr	24 hr	72 hr
col V ⁺ 60112	2.8 × 10 ⁶	2.7 × 10 ⁶	1 × 10 ⁸	1 × 10 ⁸	0.8 × 10 ⁷
col V ⁺ 23473	2.2 × 10 ⁶	1.5 × 10 ⁶	4.8 × 10 ⁵	1 × 10 ⁷	2 × 10 ⁷
col V ⁻ 41787*	3 × 10 ⁶	2.4 × 10 ⁶	1 × 10 ⁵	6.3 × 10 ⁵	3 × 10 ³
col V ⁻ 446c/1*	2.6 × 10 ⁶	2.4 × 10 ⁶	9.1 × 10 ⁴	1 × 10 ⁶	3 × 10 ²

* Strains producing unclassified colicin

Survival of *E. coli* O78 strains in the peritoneal cavity was examined by injecting 22 mice with each of two col V⁺ and two col V⁻ strains. From Table IX it is evident that after 72 hr the number of col V⁺ cells increased by about one exponent, whereas the number of col V⁻ cells decreased by 3–4 exponents.

Discussion

For a precise bacteriological diagnosis and successful epidemiological tracing, classification of species into smaller units is of great importance. Serological and phage typing of *E. coli* can only be regarded as first steps in this respect. Of other methods, detection of enterotoxin production and invasive ability may be helpful in establishing the aetiological role of members of this species.

E. coli strains associated with severe hospital infections and mild sporadic cases of enteritis, as well as those isolated from healthy infants were uniform serologically (O78: K80) and mostly uniform in phage pattern (4, 4a, 4b). Colicin-producing strains were, however, significantly more frequent among epidemic than among other strains. As regards colicin types, the difference was even more striking: 66 out of 70 colicinogenic cultures isolated from the outbreak belonged to type V. Such strains were infrequent in László Hospital and were not encountered in other hospitals and in sporadic cases. Our findings indicate that col V character in O78 strains may be associated with the severity of infection caused by them. The effect of different plasmids (Ent, col, R) on pathogenicity has been proved (9–13), but no examination of the association between phage type, colicin type and pathogenicity has been reported. Data for the correlation between colicin type and pathogenicity are rather contradictory. HAMON [14] showed 4 different colicins (I, E, B and G) in enteropathogenic *E. coli* serogroups O111, O55 and O26; V colicin, frequently detected in apathogenic *E. coli* strains, was absent from these serogroups.

BRANCHE *et al.* [15] described that in swine strains of *E. coli*, colicinogenicity was associated with certain serogroups. VASENIUS [16] showed that colicinogenic strains occurred frequently among *E. coli* isolates from sick swine. The same was described by PAPA VASSILIOU [17] and LIKHODED and KUDLAI [18] for serogroups cultured from sick infants and calves. DE ALWIS and THOMLINSON [19] showed that *E. coli* O141 strains isolated from sick swine were more frequently colicinogenic than strains of the same serogroups obtained from normal swine.

Our findings are in agreement with those of SMITH [9], who described a close connection between col V factor and pathogenicity to broiler chickens of *E. coli* O78. The plasmid nature of the gene is shown by the fact that col V production by invasive *E. coli* strains can be cured with lauryl sulphate [20].

Experiments aimed at transferring col V of our O78 strains to *E. coli* Hfr and F⁻, have failed. It may be assumed that the presence of *fi*⁺ R factor inhibited the transfer.

The present findings suggest that pathogenicity of the *E. coli* strains examined is not determined by antigens O78 or K80, but is associated with certain phage and colicin types. On the basis of transfer experiments *in vivo* [8, 21, 24] it may be assumed that certain serotypes are liable to change their pathogenicity within the body.

Our findings offer an explanation of the frequent incidence of multiple resistant *E. coli* O78 strains. About one half of the isolates carried R factor. Characterization of the R factors by their phage-restricting effect showed that the epidemic strains, containing the same R factor, probably spread later to another hospital and to a nursery.

Acknowledgement. We are indebted to Mrs. MARIANNE GYENES for skilled technical assistance.

REFERENCES

1. CZIRÓK, É., MILCH, H., MADÁR, J., SEMJÉN, G.: Acta microbiol. Acad. Sci. hung. **24**, 115 (1977).
2. KENDE, É., SERE, G., CZIRÓK, É., BÉKÉSY, Zs., JÁKÓ, Z., KORMOS, E., KUBINYI, J., MAXIMOVA, G., MIHÁLYFI, I., NYOMÁRKAI, I., NÉMEDI, L., SALFAI, G., ADAMIS, É.: Egészségtudomány **19**, 79 (1975).
3. MILCH, H., GYENES, M.: Acta microbiol. Acad. Sci. hung. **19**, 213 (1972).
4. LEWIS, M. J.: Lancet **1**, 1389 (1968).
5. FRÉDÉRICQ, P.: Rev. belge Path. méd. exp. **19**, Suppl. 4. 31 (1948).
6. GYLES, C. L., BARNUM, D. A.: J. infect. Dis. **120**, 419 (1969).
7. PESTI, L., SEMJÉN, G.: Acta vet. Acad. Sci. hung. **23**, 227 (1973).
8. SMITH, H. W., HALLS, S.: J. Path. Bact. **93**, 531 (1967).
9. SMITH, H. W.: J. gen. Microbiol. **83**, 95 (1974).
10. SMITH, H. W., LINGGOOD, M. A.: J. gen. Microbiol. **62**, 287 (1970).
11. SMITH, H. W., LINGGOOD, M. A.: J. med. Microbiol. **5**, 243 (1972).
12. SKERMAN, F. J., FORMAL, S. B., FALKOW, S.: Infect. Immun. **5**, 622 (1972).
13. EVANS, D. G., SILVER, R. P., EVANS, D. J., CHASE, D. G., GORBACH, S. L.: Infect. Immun. **12**, 656 (1975).
14. HAMON, Y.: Ann. Inst. Pasteur **96**, 614 (1959).
15. BRANCHE, W. C., YOUNG, V. M., ROBINET, H. G., MASSEY, E. D.: Proc. Soc. exp. Biol. (N. Y.) **114**, 198 (1963).
16. VASENIUS, H.: Acta vet. scand. **8**, 195 (1967).
17. PAPAVALASSIOU, J.: J. gen. Microbiol. **25**, 409 (1961).
18. ЛИКХОДЕД, В. Г.; КУДЛАИ, Д. Г.; Ж. Микробиол. **40**, 128 (1963).
19. DE ALWIS, M. C. L., THOMLINSON, J. R.: J. gen. Microbiol. **74**, 45 (1973).
20. SMITH, H. W., HUGGINS, M. B.: J. gen. Microbiol. **92**, 335 (1976).
21. GUINÉE, P. A. M.: Antonie v. Leeuwenhoek **34**, 93 (1968).
22. SALZMANN, T. C., KLEMM, L.: Proc. Soc. exp. Biol. (N. Y.) **128**, 392 (1968).
23. JAROLMEN, H.: Ann. N. Y. Acad. Sci. **182**, 72 (1971).
24. SMITH, M. G.: J. Hyg. (Lond.) **75**, 363 (1975).

Address of the authors:

HEDDA MILCH
National Institute of Hygiene
H-1966 Budapest, P.O.B. 64, Hungary

ÉVA CZIRÓK, JÁNOS MADÁR
Pest County Public Health Station
H-1428 Budapest, P.O.B. 55, Hungary

GÁBOR SEMJÉN
Veterinary Research Institute, Hungarian Academy of Sciences
H-1581 Budapest, P.O.B. 18, Hungary

PHENOTYPIC CORRECTION OF NONSENSE MUTATION CARRYING NON-CONVERTING PE5 PHAGES IN SHIGELLA FLEXNERI WITH SUPPRESSOR GENE

I. FINANCSEK and I. KÉTYI

Institute of Microbiology, University Medical School, Pécs

(Received July 14, 1976)

(i) Phenotypic suppression by aminoglycoside antibiotics of a polyauxotrophic *Shigella flexneri* var. Y strain on partially completed minimal medium has shown that its Thr dependence is associated with nonsense mutation. Induced Thr⁺ revertants selected from the culture yielded clones correcting the lytic cycle of nonsense T4 mutant phages. Transfer of R1_{am} plasmid to these clones carrying a nonsense mutation of ampicillin resistance was performed. In this manner a *S. flexneri* var. Y derivative was isolated which, on the basis of the phenotypic correction of T4 phages and R1_{am} factor, proved to be a suppressor positive clone. (ii) From phage PE5 responsible for conversion of type antigen V, mutants were isolated that had lost their converting capacity. Selected Sup⁺ and control Sup⁻ strains were treated with the mutant phages and examined for the appearance of type antigen V. Three phage mutants were found to induce antigen conversion only in Sup⁺ strains. (iii) The data suggest that, at least with phage PE5, the information for type antigen conversion is carried by phage genome.

The basic problem of the mechanism of lysogenic conversion is whether the information is of phage genome origin or the prophage only derepresses a bacterial gene. Our earlier results [1] were suggestive of the former mechanism: we isolated a phage (P90) which converted a modified antigen of IVc. In further experiments we examined at permissive and non-permissive temperatures the glycosyl-transferase activity of membrane preparations of strains lysogenized with thermosensitive PE5 mutants. The facts that enzyme activity at non-permissive temperatures was low or nil and that these phage mutants were somewhat different in behaviour [2] also indicated a type antigen information associated with phage genome.

The present examinations were based on the assumption that if non-converting phage mutants which have lost their converting capacity in consequence of nonsense mutation, can be isolated, they may be subjected to phenotypic correction in a strain carrying suppressor gene. These data would prove the phage genome origin of the conversion mechanism.

Materials and methods

Bacterial strains. *S. flexneri* var. Y strain UP518 is maintained in the collection of this institute. Suppressor gene-carrying derivative was obtained from *S. flexneri* var. Y strain NTCC 4839; from this by EMS mutagenesis, a Nia⁻Met⁻Thr⁻Leu⁻ polyauxotrophic mutant (UP 156) was selected. Thr⁺ revertants of mutant UP 156 yielded strain Su2 (Sup⁺). For

nonsense phages T4, suppressor strains *Escherichia coli* K12 CA165 (ochre) and CA266 (amber) served as relevers [3]. Plasmid R1_{am} was carried by *E. coli* K12 derivative UB1924 [4].

Bacteriophages. Phage PE5 isolated by us earlier [5] causing conversion to type antigen V and its non-converting mutants were used.

Sup⁺ *S. flexneri* strains were checked with ochre (eL1) and amber (eL1a) nonsense mutants of phage T4. Mutant T4-eL1 carries the nonsense mutation in the lysozyme gene; mutant T4-eL1a is its derivative; consequently, these phages are unable to produce lytic cycle in Sup⁻ strains [3].

Culture media. As complete medium, Lennox's broth (LB) and its solid form made with 1.5% Difco agar (LB agar) or with 0.6% Difco agar (top layer) were used. The broth contained tryptone, 10 g; yeast extract, 5 g; NaCl, 5 g; water, 1000 ml, pH 7.2 [6].

Davis's minimal medium [7] was modified to contain: glucose, 8 g; NaCl, 2 g; MgSO₄, 0.05 g; (NH₄)₂SO₄, 1 g; KH₂PO₄, 5.44 g; K₂HPO₄, 16.49 g; water, 1000 ml. The ingredients (analytical grade) were dissolved in 100 ml water, the pH was adjusted to 7.1 and the solution was passed through Seitz filter. The stock salt solution was added to autoclaved agar base, supplemented with amino acids, etc. (20 µg/ml) and poured in plates.

Preparation of phage lysates, testing of phage sensitivity, lysogenization and examination of lysogenic conversion. Phage lysates were obtained by washing off overlayers showing semi-confluent lysis. After adding chloroform, the lysates were centrifuged; the supernatant contained 10⁹–10¹⁰ p.f.u. per ml.

Screening test for phage sensitivity was made by streaking bacterial cultures diagonally to lines of the phage lysates dried on LB agar. Quantitative examinations were performed by the overlayer technique. Comparison of the titres (p.f.u./ml) was expressed in e.o.p. values.

For lysogenization the bacteria were spread on agar plate and spotted with phage lysates. Surviving bacteria growing in the lytic zones were streaked on agar and isolated colonies were picked up. Lysogeny was tested by transferring a substantial amount of bacteria onto an overlayer containing the tested culture (UP518). After incubation a definite lytic zone developed around the bacterial spot.

For testing lysogenic conversion only lysogenic clones (showing release of phages) were used. Slide agglutination was performed with *S. flexneri* absorbed serum V prepared in this institute.

Antibiotics. To examine phenotypic correction by aminoglycoside antibiotics, crystals of the substances or disks 5 mm in diameter dipped into solutions (1 mg/ml) were used: streptomycin (EGYT, Budapest); kanamycin (Canamycin-SO₄ V/O, USSR); neomycin (Mycerin, V/O, USSR); erythromycin (Erythrocin, Abbot Labs, UK) served as control. For R1_{am} transfer, the following substances were used: streptomycin (EGYT, Budapest); chloramphenicol (Chlorocid, EGYT, Budapest); carbenicillin (Microcillin, Bayer, GFR); and ampicillin (Ampicillin-Na, Pharmachin, Bulgaria).

Phenotypic correction for aminoglycoside antibiotics was made with the method of WHITEFIELD *et al.* [8].

R factor transfer and testing. For checking the presence of the suppressor gene, R1_{cm} factor was used [4]. Strain UB1924 pro⁻his⁻trp⁻ (R1_{am}) proved streptomycin (100 µg/ml) and chloramphenicol (10 µg/ml) resistant, kanamycin sensitive and carbenicillin and ampicillin sensitive on Sup⁻ strains. The *amp* gene carried nonsense mutation. In transfer experiments, strain K12 UB1924 was used as donor, strain UP156 Sup⁻ and its assumed Sup⁺ derivatives as recipient. Selection was made on completed minimal medium in the presence of streptomycin (100 µg/ml) and chloramphenicol (10 µg/ml). R⁺ *Shigella* derivatives were reisolated on the same medium.

Carbenicillin and ampicillin resistance was determined by single cell technique, on LB agar plates containing the antibiotics at different concentrations [4]; each plate was inoculated with 10³–10⁴ cells so as to obtain isolated colonies.

Mutagenesis. Polyauxotrophic mutants of *S. flexneri* NTCC 4839 were induced with ethyl methane sulphonate (EMS) as described by CLARK [9].

Thr⁺ revertants were obtained from strain UP156 by *N*-methyl-*N'*-nitroso-*N*-nitroguanidine treatment using the optimal method of ADELBERG *et al.* [10].

Phage mutants PE5 defective in converting ability were induced by hydroxylamine [11].

The nomenclature recommended by DEMEREC *et al.* [12] was used.

Results

1. *Isolation of S. flexneri* var. Y derivative carrying suppressor gene and checking of Sup^+ character. Suppressor mutants can easily be isolated by revertants of strains having acquired dependence by nonsense mutation. These are partly real, partly suppressor revertants.

The experiments were started with *S. flexneri* var. Y strain NTCC 4839 sensitive to phage PE5; the original strain was Nia^- . EMS mutagenesis yielded several mutants, from which polyauxotrophic mutants were derived. One of the latter showed not only Nia^- dependence, but also $Met^-Thr^-Leu^-$ characters.

Mutant UP156 was streaked onto (partly) completed minimal medium devoid of methionine or threonine or leucine. Then crystals of antibiotics or disks containing these substances were placed on the medium; erythromycin served as control. Crystals and disks both gave evaluable results. On the plate shown in Fig. 1 streptomycin and neomycin crystals and kanamycin and erythromycin disks were placed.

Figure 1 demonstrates a markedly increased growth at the border of aminoglycoside inhibition zones and the bacterial lane; this growth is absent around the erythromycin disk. The phenomenon is associated with phenotypic

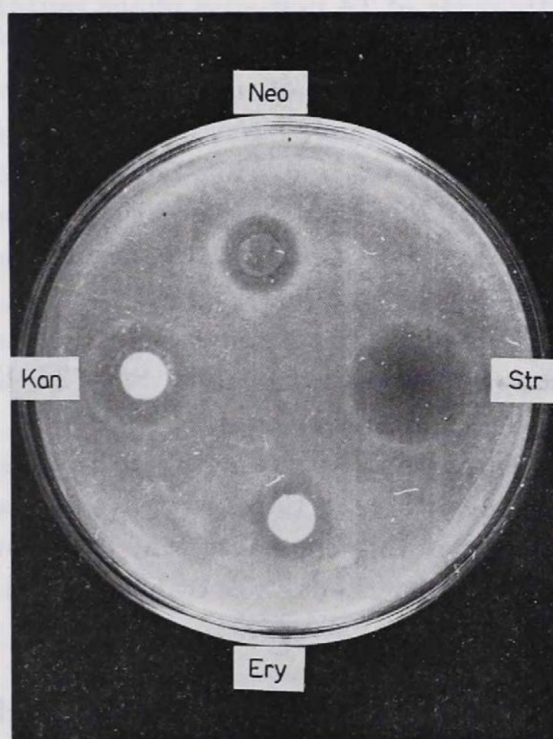


Fig. 1. Growth of *S. flexneri* var. Y mutant UP156 $Nia^-Met^-Thr^-Leu^-$ on threonine-free minimal medium with neomycin and streptomycin crystals and erythromycin and kanamycin disks placed on the surface. The marked ring-shaped growth on the border of neomycin, streptomycin and kanamycin inhibition zones indicate phenotypic correction by aminoglycoside antibiotics

correction of dependence and appeared distinctly on threonine-free medium, indicating that Thr⁻ mutation is of nonsense character.

In the next step, about 200 Thr⁺ revertants were isolated by NTG mutagenesis. As mentioned above, part of Thr⁺ revertants are assumed to be suppressor ones, i.e., in threonine biosynthesis a phenotypic correction has taken place. The 200 Thr⁺ revertants were examined by "cross-streak" method with T4-eL1 (ochre) and T4-eL1a (amber) nonsense phages using T4 wild-type phage as control and, in view of the purpose of the experiment, with the lysate of phage PE5.

For quantitative assay, phage T4-eL1 (ochre nonsense) was propagated on strain ochre Sup⁺CA165, phage T4-eL1a (amber nonsense) on strain amber Sup⁺CA266, and lysates were prepared on the two strains with wild-type T4 phage, too. With these phage lysates quantitative tests were carried out on the six UP156 Thr⁺ revertants assumed to be Sup⁺, on the original Sup⁻ UP156 strain and on the two Sup⁺ K12 relevator strains. Results are shown in Table I.

The fact that UP156 and its Thr⁺ revertants failed to exert any appreciable restriction on wild-type phage T4, greatly facilitated to interpret the results.

It is evident that, in agreement with the results of the preliminary examinations, the lytic cycle of T4 nonsense phage mutants takes place in the six Thr⁺ revertants. From the phenotypic correction of this cycle compared to standard K12 Sup⁺ derivatives it may be assumed that UP156 Thr⁺ derivatives Su2, Su3, Su12 and Su22 carry ochre suppressor, while Su13 and Su19 carry amber suppressor mutation. This, however, is somewhat invalidated by the fact that these *Shigella* strains are not isogenic with *E. coli* K12.

In view of the above consideration, we wished to test the suppressor character of our clones by other methods. Use of the R1_{am} factor of KENNEDY and CROWLESMITH [4] seemed to offer a simple solution. As an effect of induced mutation, this R factor, carrying originally Str Chl Kan Amp resistance, had exhibited a nonsense mutation in the ampicillin resistance gene and became sensitive to kanamycin. Accordingly, in Sup⁻ strains the ampicillin resistance fails to appear, whereas Sup⁺ strains are capable of phenotypic correction. On the basis of this consideration, by means of the procedures described in Materials and methods, we transferred R1_{am} factor to strain UP156 Sup⁻ and to its Thr⁺ derivatives assumed to carry suppressor mutation. Results are shown in Table II.

It is seen that only Su2 revertant behaved similarly to Sup⁺ strains of KENNEDY and CROWLESMITH (resistant to ampicillin and highly resistant to carbenicillin, which is more susceptible to lactamase than the former [4]).

We have not examined further the reason for the ampicillin sensitivity and low carbenicillin resistance of other Thr⁺ revertants assumed to be Sup⁺.

Table I

Examination of S. flexneri var. Y mutant UP156 Thr⁻ and its assumed suppressor revertants with wild-type and nonsense phages T4

Strains	Bacteriophages							
	T4 266 (wild)		T4 165 (wild)		eL1a 266 (amber)		eL1 165 (ochre)	
	p.f.u. per ml	e.o.p.	p.f.u. per ml	e.o.p.	p.f.u. per ml	e.o.p.	p.f.u. per ml	e.o.p.
CA266 am <i>sup</i> ⁺	8.6×10^9	<i>I</i>	1.9×10^9	<i>I</i>	2.6×10^{10}	<i>I</i>	1.1×10^5	1.0×10^{-5}
CA165 oc <i>sup</i> ⁺	9.9×10^9	<i>I</i>	3.9×10^9	<i>I</i>	2.8×10^{10}	<i>I</i>	1.7×10^{10}	<i>I</i>
UP156 Sup ⁻	2.0×10^8	4.9×10^{-1}	1.1×10^8	3.5×10^{-1}	$< 2 \times 10^2$	$> 10^{-8}$	$< 2 \times 10^2$	$> 10^{-8}$
Su2 Thr ⁺	1.0×10^{10}	<i>I</i>	2.3×10^9	<i>I</i>	4.6×10^9	5.6×10^{-1}	2.7×10^8	6.3×10^{-1}
Su3 Thr ⁺	1.2×10^{10}	<i>I</i>	2.9×10^9	<i>I</i>	2.3×10^{10}	<i>I</i>	1.0×10^9	1.7×10^{-1}
Su12 Thr ⁺	1.0×10^{10}	<i>I</i>	3.8×10^9	<i>I</i>	2.6×10^{10}	<i>I</i>	3.3×10^9	5.0×10^{-1}
Su13 Thr ⁺	1.0×10^{10}	<i>I</i>	2.3×10^9	<i>I</i>	4.2×10^9	6.1×10^{-1}	6.0×10^3	2.8×10^{-6}
Su19 Thr ⁺	8.4×10^9	<i>I</i>	2.0×10^9	<i>I</i>	1.2×10^9	2.1×10^{-2}	4.8×10^4	3.5×10^{-5}
Su22 Thr ⁺	1.1×10^{10}	<i>I</i>	3.7×10^9	<i>I</i>	2.7×10^{10}	<i>I</i>	5.9×10^9	2.8×10^{-1}

Table II

Carbenicillin and ampicillin sensitivity of derivatives of S. flexneri var. Y. mutant UP156 assumed to be Sup⁺, carrying the Rl_{am} plasmid

Strains	Carbenicillin μg/ml	Ampicillin 20 μg/ml
UP156 Sup ⁻ (Rl _{am})	5	sensitive
Su2 (R ⁻)	5	sensitive
Su2 (Rl _{am})	> 500	resistant
Su3 (Rl _{am})	50	sensitive
Su12 (Rl _{am})	100	sensitive
Su13 (Rl _{am})	< 20	sensitive
Su19 (Rl _{am})	100	sensitive
Su22 (Rl _{am})	100	sensitive

Resistance was tested by "single cell" method with 10^3 – 10^4 cells per plate allowing the growth of isolated colonies

Results with derivative Su2 have been considered satisfactory; the lack of isogeny explains in these experiments, too, the less effective correction.

Derivative Su2 was subjected to some control examinations and its Thr independence was again confirmed. Testing of adsorption of phage PE5 showed that this derivative, although inferior to strain UP156 ($K_{156} = 2.96 \times 10^{-9}$ and $K_{Su2} = 2.4 \times 10^{-9}$; 100% and 81%, respectively) was still suitable for lysogenization. Finally, it was checked whether Su2 produces type specific antigen V on lysogenization with wild-type phage PE5.

2. *Isolation of PE5 phage mutants defective in antigen-converting capacity and examination of phenotypic correction of antigen conversion in S. flexneri var. Y strain Su2 assumed to carry ochre suppressor mutation.* Treatment of wild-type phage PE5 with hydroxylamine results in the appearance of about 10% clear-plaque mutants showing the effectivity of the mutagenesis. After mutagenization, the lysate was layered onto LB agar plates at dilutions giving isolated plaques. After 48–72 hr incubation colonies within the plaques were picked up and tested for lysogeny. Clones found lysogenic were tested for the presence of antigen V.

The majority of clones from 350 plaques was lysogenic; in 19 of them the converted antigen V was absent. Phages released from the 19 clones were isolated, purified by three reisolations of single plaques, then lysates giving about 10^9 p.f.u./ml were prepared.

Clone Su2, assumed to carry a suppressor gene, and control strain UP518, showing a high rate of convertibility, were lysogenized with the above preparations.

Table III

Lysogenic conversion in S. flexneri var. Y Sup⁻ and Sup⁺ strains by PE5 wild-type phage and its mutants defective in converting ability

Phages	No. of antigen convertants with antigen V per lysogenic clones	
	UP518 Sup ⁻	UP518 Sup ⁺ ("Su2")
PE5 wild-type	72/100	64/100
PE5-No. 5	0/100	6/92
PE5-No. 11	0/100	10/120
PE5-No. 28	0/100	26/116

In these experiments three phage mutants were isolated which exerted a conversion to antigen V in Su2 but not in UP518 (Table III).

It is evident that phage mutants Nos 5, 11 and 28, defective in antigen-converting capacity, had undergone nonsense mutation subjected to a phenotypic correction in derivative Su2. As a result, type antigen V appeared in the host cell.

Discussion

Our experiments have shown that aminoglycoside antibiotics exert phenotypic correction for auxotrophic mutants of shigellae, too. In this manner we were able to determine the dependence based on nonsense mutation. Selection of suppressor mutants from the isolated Thr⁺ revertants was somewhat difficult, since the control systems available were not isogenic. Yet, by the use of T4 phages carrying nonsense mutation, we could isolate six derivatives exerting suppressive correction on T4 mutant phages in a degree comparable to K12 suppressors.

At transferring R1_{am} factor (also non-isogenic), the results could be checked again. Out of the six mutants correcting the lytic cycle of defective phages carrying T4 nonsense mutation (and assumed to be Sup⁺), only one was ampicillin resistant and highly carbenicillin resistant. The rest of the assumably Sup⁺ derivatives exhibited some degree of resistance to carbenicillin, which indicates an at least partial phenotypic correction of β -lactamase. The seemingly contradictory data may be attributed to the use of non-isogenic systems.

It is, however, evident that mutant Su2 was suitable for the correction of three different PE5 mutants defective in inducing the synthesis of type antigen V in Sup⁻ strains.

As mentioned in the introduction, the purpose of these investigations was to obtain new data for the synthesis of lysogenic conversion-associated type antigens in *S. flexneri*, that is, to prove that the information for type antigen conversion is carried by the phage genome.

S. flexneri seems especially suitable for studying such problems. The basic type of the subgenus is known to be variant Y, which contains the group antigen "3, 4". The existence of different serotypes characterized by type specific antigens I, II, III, IV and V and group antigens 6 and 7, 8 is a result of lysogenic conversion [13-16]. Except group antigen 6, which is an acetylated form of antigen 3,4 [16], all these antigens are defined by α -glycosyl secondary side chains at different locations [17, 18]. Since one common host is differentiated into serotypes by various phages, the hypothesis that phage genomes informing glycosyl transferases of different specificity are involved, seems more probable than the assumption that prophages act only as derepressors of a series of repressed bacterial genes.

Our interest in the problem dates back to the isolation of phage P90, which converted an antigen different from the one acted upon by phage ϕ IV₁ of ISEKI and HAMANO [13]. The antigen in question (IV_c) showed an a, b, -a, c relationship within the same host.

JANKOWSKI *et al.* [19] have shown that the glucose donor for *S. flexneri* O antigens containing α -glycosyl group is polyisoprenol-phosphate-glucose and that in the absence of glycosylated O antigen no lipid-bound glucose is produced. In their further work they demonstrated a similar finding for the membrane fraction of a non-lysogenic *S. flexneri* var. Y strain and of its counterpart lysogenized with our phage PE5. In an earlier study [2] we analysed the same phenomenon with thermosensitive converting mutants of phage PE5 at permissive and non-permissive temperatures.

Analysis of phage-induced antigenic changes in *Salmonella* preceded the corresponding studies in *Shigella*. STOCKER and MÄKELÄ [21] suggested that a modification of LPS is usually associated with the action of phage genes. A classical example is *Salmonella* group E, where epsilon 34 phage is responsible for the biosynthesis of the α -glycosyl terminal group. HARADA *et al.* [22] in 1964 already described an epsilon 34 phage mutant which had lost its antigen-converting ability. WRIGHT and BARZILAI [23] distinguished two groups of non-converting mutants of epsilon 34 phage; one of them failed to promote glucose transfer to lipid phosphate, the other was deficient in transferring glucose from the lipid. Concluding from the above data, we consider that the hypothesis of phage genome information in *S. flexneri* antigen conversion has been proved.

Our earlier and the present data seem to confirm that PE5 phage-induced conversion of type antigen V is associated with phage genes. The same probably holds true for other *S. flexneri* antigen determinants.

Acknowledgements. We are indebted to Dr. W. H. McCLAIN (Department of Bacteriology, University of Wisconsin, Madison, Wisconsin, U.S.A.) for T4 wild-type and nonsense phage mutants and relevator strains, and to Dr. C. KENNEDY (ARC Unit of Nitrogen Fixation, University of Sussex, Brighton, U.K.) for R1_{am} plasmid-carrying UB1924 strain.

REFERENCES

1. KÉTYI, I., VERTÉNYI, A., FINANCSEK, I.: *Acta microbiol. Acad. Sci. hung.* **18**, 47 (1971).
2. FINANCSEK, I., KÉTYI, I., SASAK, W., JANKOWSKI, W., JANCZURA, E., CHOJNACKI, T.: *Infect. Immun.* **14**, 1290 (1976).
3. SEIDMAN, J. G., COMER, M. M., McCLAIN, W. H.: *J. Mol. Biol.* **90**, 677 (1974).
4. KENNEDY, C., CROWLESMITH, I.: *Molec. gen. Genet.* **138**, 359 (1975).
5. KÉTYI, I., VERTÉNYI, A., FINANCSEK, I.: *Acta microbiol. Acad. Sci. hung.* **19**, 159 (1972).
6. LENNOX, E. S.: *Virology*, **1**, 190 (1955).
7. KÉTYI, I.: *Acta path. microbiol. scand.* **75**, 104 (1969).
8. WHITEFIELD, H. J. JR., MARTIN, R. G., AMES, B. N.: *J. molec. Biol.* **21**, 335 (1966).
9. CLARK, A. J.: *J. Cell Physiol.* **70**, 165 (1967).
10. ADELBURG, E. E., MANDEL, M., CHEIN CHING CHEN, G.: *Biochem. Biophys. Res. Comm.* **18**, 788 (1965).
11. LYONS, L. B., ZINDER, N. D.: *Virology* **49**, 45 (1972).
12. DEMEREC, M., ADELBURG, E. A., CLARK, A. J., HARTMAN, P. E.: *Genetics* **54**, 61 (1966).
13. ISEKI, S., HAMANO, S.: *Proc. Jap. Acad. Sci.* **35**, 407 (1959).
14. GIAMMANCO, G.: *Ann. Inst. Pasteur* **114**, 63 (1968).
15. GIAMMANCO, G., NATOLI, D.: *Ann. Inst. Pasteur* **117**, 16 (1969).
16. GEMSKI, P. JR., KOELTZOW, D. E., FORMAL, S. B.: *Infect. Immun.* **11**, 685 (1975).
17. SIMMONS, D. A. R.: *Bacteriol. Rev.* **35**, 117 (1971).
18. SELTMANN, G.: *Z. allg. Mikrobiol.* **12**, 497 (1972).
19. JANKOWSKI, W., CHOJNACKI, T., JANCZURA, E.: *J. Bact.* **112**, 1420 (1972).
20. JANCZURA, E., JANKOWSKI, W., CHOJNACKI, T.: *Abstr. Comm. 9th Meet. FEBS s6 b23*, p. 229 (1974).
21. WEINBAUM, G., KADIS, S., AJL, S. J.: *Microbiological Toxins*. In: STOCKER, B. A. D., MÄKELÄ, H. P. (eds): *Bacterial Endotoxins*. Vol. 4, Chapter 10. Academic Press, New York 1971, pp. 369-438.
22. HARADA, K., KAMEDA, M., SUZUKI, M., MITSUHASHI, S.: *Jap. J. Microbiol.* **8**, 125 (1964).
23. WRIGHT, A., BARZILAI, N.: *J. Bact.* **105**, 937 (1971).

Address of the authors:

ISTVÁN FINANCSEK, IVÁN KÉTYI
Institute of Microbiology, University Medical School
Szigeti út 12, H-7643 Pécs, Hungary

DISTRIBUTION OF ^{32}P -LABELLED ENDOTOXIN IN THE FROG

T. SZILÁGYI and GY. DESEŐ

*Institute of Pathophysiology, University Medical School,
Debrecen*

(Received July 16, 1976)

Distribution and elimination of ^{32}P -labelled endotoxin were studied in normal frogs and in frogs pretreated with a high dose of unlabelled endotoxin 24 hr previously. Like in other animals, the spleen and the liver of the frogs were found the most active R.E.S. organs. The alimentary tract too exhibited considerable R.E.S. activity. Pretreatment with unlabelled endotoxin resulted in a decrease of ^{32}P -endotoxin uptake by the liver, the spleen and the alimentary tract. Evidence was obtained that the well-known effects of endotoxin on the R.E.S. of the mammals can be demonstrated in the frog as well.

Besides the immunofluorescent techniques based on the antigenicity of endotoxin [1, 2], isotope labelling proved suitable for tracing endotoxin in different organs, tissues and cells [3–20]. Radioisotopes such as ^{131}I [3, 4], ^3H [5, 8], ^{14}C [9], ^{51}Cr [10–17], ^{32}P [18–20] have been used for marking endotoxin. The method of labelling and the choice of the radioactive marker were of crucial importance in studies *in vivo*, therefore they have to be taken into account when evaluating experimental results [9–11, 13–20].

Unlike mammals, cold-blooded vertebrates are extremely resistant to endotoxin [21, 22]. However, both our data [23, 24] and those of other investigators [22, 25, 26] indicate that the effects of endotoxin can be demonstrated in the frog as well. On the basis of these observations we decided to investigate the distribution of endotoxin in the frog. Studying the subject seems to be justified also by the fact that no relevant data for other species of cold-blooded vertebrates have been reported.

This paper reports on the distribution and the elimination of an endotoxin preparation labelled with ^{32}P in normal frogs and in frogs pretreated with unlabelled endotoxin.

Materials and methods

Adult frogs (*Rana esculenta* L.) of both sexes were used.

Endotoxin was prepared from *Escherichia coli* O124 strain by the method of BOIVIN and MESROBEANU [27]. The biological activity of endotoxin ($\text{DL}_{50}/24$ hr intraperitoneally) was determined in mice relatively resistant to endotoxin. One ml of ^{32}P -labelled endotoxin gave 5×10^5 counts/min. Considering the efficiency of the counter the specific activity cor-

responded to 5 $\mu\text{Ci/ml}$. Unlabelled endotoxin was administered into the dorsal lymph-sack of the frog while labelled endotoxin was given intraperitoneally, in a volume of 1 ml. The dose/animal of both unlabelled and labelled endotoxin was double the mouse LD_{50} calculated for the frog's total body weight. The animals were killed at different intervals after treatment and the activity of the organs and tissues was measured with a GM counter [19]. The values obtained were extrapolated to zero time (the time of endotoxin administration) by correction with the corresponding decay factor.

Results

Figure 1 shows the distribution of ^{32}P -endotoxin in the control animals, i.e. those that had not been pretreated with unlabelled endotoxin. Values are expressed in cpm/g wet weight. Like in mammals, the highest specific activity was found in the spleen and the liver. Activity in the gastrointestinal tract was somewhat lower, but it decreased with time similarly as in the liver and the spleen. Activity of the blood fell 6 hr after the administration of ^{32}P -endotoxin. There was a rise at 24 hr followed by a gradual decrease. Activity of the lung decreased after the 2nd hour following administration. Activity in the kidneys and the skin remained almost unchanged during the entire observation period, while in the skeletal muscles it slowly increased with time.

The cpm/organ values are represented in Fig. 2. Comparison of the data in Fig. 1 with those of Fig. 2 revealed a striking difference as far as the spleen

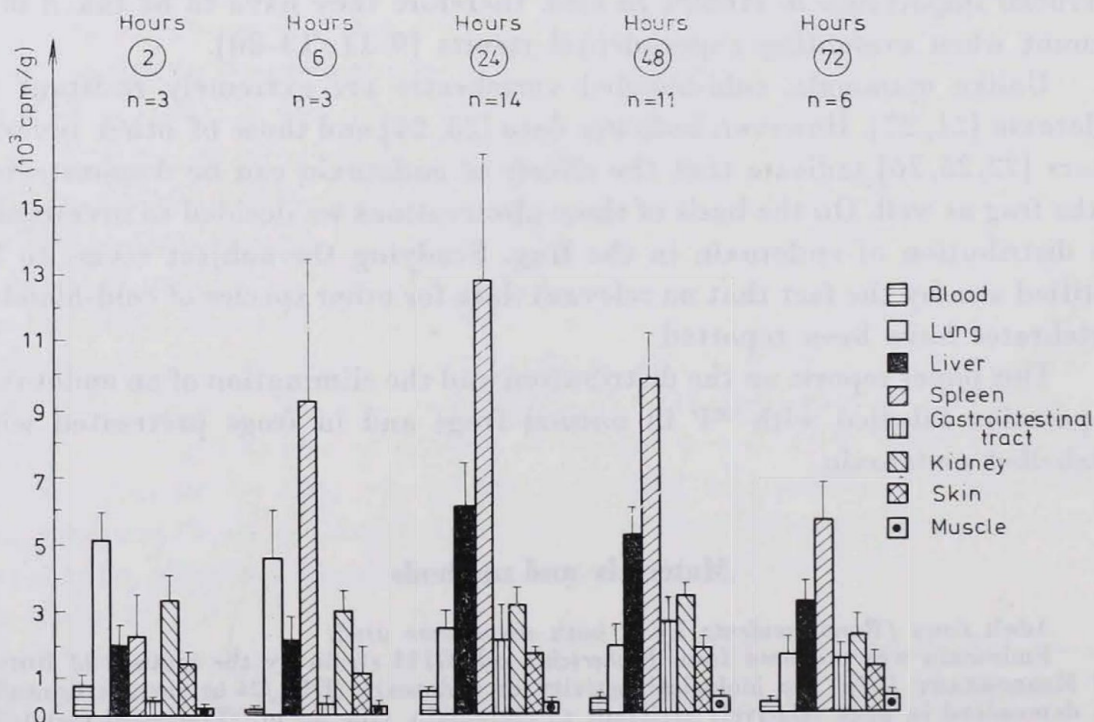


Fig. 1. ^{32}P -endotoxin uptake by different organs and tissues of normal frogs (cpm/g wet weight)

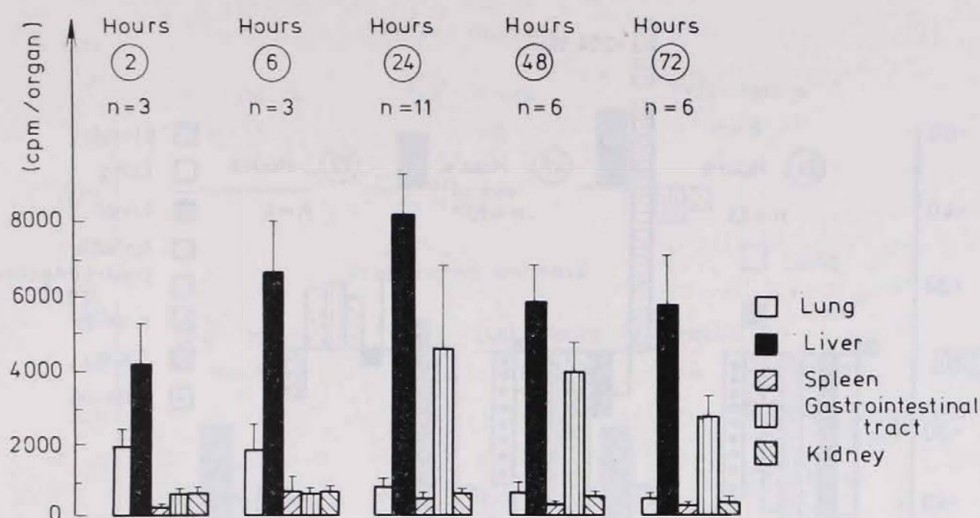


Fig. 2. Storage of ^{32}P -endotoxin in different organs of normal frogs (cpm/organ)

which exhibited a considerable specific activity, seemed nearly unimportant in storage. A large proportion of endotoxin was taken up by the liver and a smaller part by the gastrointestinal tract. The isotope content of the lungs gradually decreased with time, while that of the kidneys remained almost unchanged. Although not indicated in the figures, the endotoxin taken up by the blood, the skin and the skeletal muscles amounted to an important part of the whole stored amount. Apart from a second peak noted at 24 hr, the importance of blood in ^{32}P storage decreased with time while the skeletal muscles became increasingly important. The high and practically constant activity level in the skin corresponded to a large amount of isotope, the wet weight of the skin being 9 to 10% of the body weight of the frog.

In Fig. 3 the control animals are compared with those pretreated with a high dose of unlabelled endotoxin. Twenty-four hours after ^{32}P -endotoxin administration, activity in the liver and the spleen was about 60% lower in the pretreated animals than in the controls. The difference decreased in almost every tissue by the 48th hr. The blood was an exception, its activity being much higher in the pretreated frogs, as noted particularly at 48 hr. Seventy-two hours after ^{32}P -endotoxin injection the tissues showed slightly higher activity values in the pretreated animals than in the controls. The cpm/organ values for the pretreated frogs were also considerably lower than those for the controls 24 hr after the injection of labelled endotoxin. The difference decreased by the 48th hr and became inverse at 72 hr (Fig. 4).

In Fig. 5 the pretreated and the control animals are compared in respect of organ weights. Considering that the animals belonged to the same weight group, the values are uniformly expressed in terms of the organ weights of the controls measured 24 hr after the administration of ^{32}P -endotoxin. The

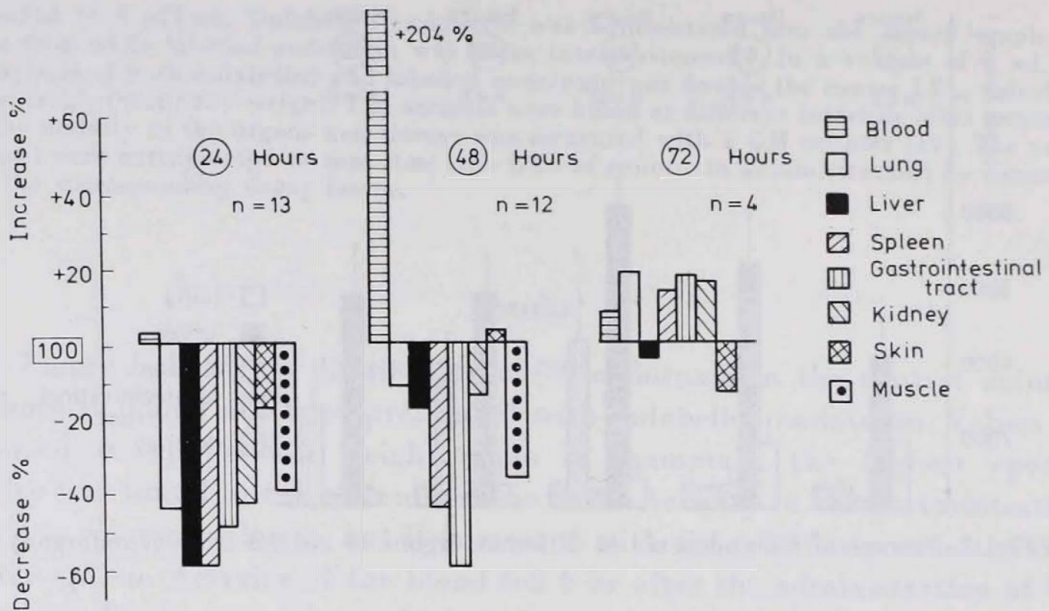


Fig. 3. ^{32}P -endotoxin activity in different tissues and organs as measured 24, 48 and 72 hr after the administration of labelled endotoxin (cpm/g wet weight). Animals were pretreated with a high dose of unlabelled endotoxin 24 hr prior to the administration of ^{32}P -endotoxin; 100% = 24, 48 and 72 hr values in Fig. 1

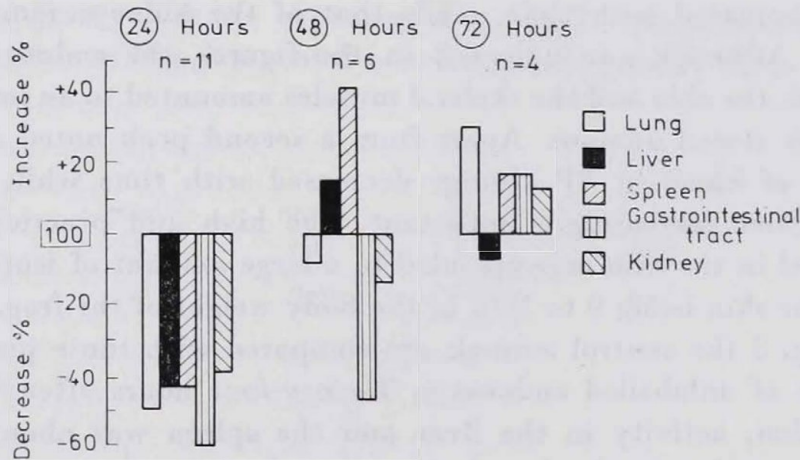


Fig. 4. ^{32}P -endotoxin activity in different tissues and organs as measured 24, 48 and 72 hr after administration of labelled endotoxin (cpm/organ). Animals were pretreated with a high dose of unlabelled endotoxin prior to the administration of labelled endotoxin; 100% = 24, 48 and 72 hr values in Fig. 2

injection or injections of endotoxin were followed by a striking increase in liver and spleen weight; e.g. the wet weight of the liver increased by 30 to 70%. Regardless of the number of endotoxin injections, the highest values were found 72 hr after the first injection, particularly in the liver and the spleen. The changes in weight were more pronounced in the animals treated with endotoxin on two occasions.

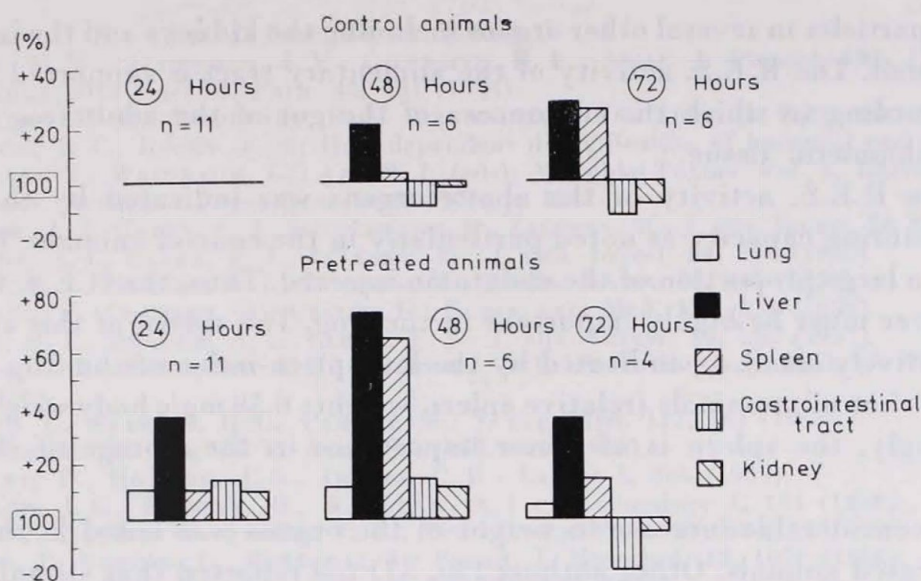


Fig. 5. Changes in organ weight after endotoxin treatment. Control animals were given endotoxin once; 24 hr values = 100%. Pretreated animals were given endotoxin on two occasions, 24 and 48 hr prior to the 24 hr observation

Discussion

Cold-blooded animals including the frog are known to be remarkably resistant to the lethal effect of endotoxin [21, 22], but in a previous study we could show that the endotoxin caused a full activation of the blood coagulation system (a phylogenetically primitive phenomenon) also in *Rana esculenta*, and *E. coli* endotoxin treatment elicited consumption coagulopathy and neutropenia in these animals [23]. The blood-lymph barrier was also affected by the endotoxin [24]. Other investigators reported the complement depleting activity of endotoxin in the serum of the frog [25]. Endotoxin caused thrombopenia in the frog *Rana pipiens* and a considerable increase in the electric activity of the isolated skin. Inactivation of endotoxin by the serum is a phylogenetically very old phenomenon which had first appeared in the cartilaginous fishes [29].

Considering the above data it was not surprising that the well-known effects of endotoxin on the R.E.S. of mammals [14, 19, 30, 31] could be demonstrated in the frog as well. Like in mammals [1-4, 8, 9, 11-13, 15, 18, 19, 31] the spleen and the liver of the frog were found the R.E.S. organs most active in the uptake and storage of endotoxin. India ink particles injected into the frog *Rana pipiens* were detected in the liver and the spleen [32] and in the present study the alimentary tract too, was observed to have a considerable storing capacity. A similar observation was made by FOXON and ROWSON [33] who found that thorium dioxide introduced into the dorsal lymph-sack of the frog *Rana temporaria* was removed from the circulation by the phagocytes of the spleen and the liver. Using a high dose of endotoxin they found thorium

dioxide particles in several other organs including the kidneys and the intestinal wall as well. The R.E.S. activity of the alimentary tract is supported by data [34] according to which the submucosa of the gut of the adult frog consists of granulopoietic tissue.

The R.E.S. activity of the above organs was indicated by their high specific storing capacity as noted particularly in the control animals. The liver took up a large proportion of the endotoxin injected. Thus, the R.E.S. function of the liver must be highly important in the frog. The spleen of this animal is comparatively small as indicated by the low spleen-index of the frog as compared to that of mammals (relative spleen weight: 0.58 mg/g body weight [35]). Accordingly, the spleen is of minor importance in the storage of ^{32}P -endotoxin.

A considerable increase in weight of the organs was noted in the endotoxin-treated animals. Other authors [36, 37] too reported that stimulation of the R.E.S. resulted in the proliferation of macrophages with increased metabolic activity. The organs containing such cells rapidly increase in weight along with increasing blood flow.

Radioactivity of the blood of the control animals showed a biphasic pattern. After a transitory fall noted at 6 hr, the activity rose 24 hr after the administration of ^{32}P -endotoxin. The phenomenon may be explained by the partial liberation of the stored endotoxin and/or by the liberation of $^{32}\text{PO}_4$ from endotoxin. In the frog, the changes in the ^{32}P -endotoxin content of the lung were similar to those observed in the mouse [15], i.e. the specific activity decreased the changes being almost parallel to those in the blood. The comparatively slow decline in the specific activity of the skin and the kidneys may have been due to the elimination of ^{32}P (^{32}P -endotoxin). There is a possibility that the serum alkaline phosphatase splits $^{32}\text{PO}_4$ from endotoxin in the frog as well [18, 20]. Excretion of $^{32}\text{PO}_4$ with urine and perspiration may be the most important ways of elimination of ^{32}P . The slow but definite increase in the isotope content of the skeletal muscles speaks also for the liberation of anorganic $^{32}\text{PO}_4$. This increase is likely to be due to the utilization of $^{32}\text{PO}_4$ in the intensive creatine-phosphate metabolism.

Like in mammals [14, 19, 31] treatment of the frog with a high dose of endotoxin resulted in R.E.S. blockade 24–48 hr after the endotoxin injection. The changes in the activity of the tissues of pretreated animals suggest a biphasic R.E.S. effect of endotoxin in the frog as well. Thus, it seems likely that the first blockade period is followed by a period of increased R.E.S. activity.

REFERENCES

1. CREMER, N., WATSON, D. W.: *Proc. Soc. exp. Biol. (N. Y.)* **95**, 510 (1957).
2. RUBENSTEIN, H. S., FINE, J., COONS, A. H.: *Proc. Soc. exp. Biol. (N. Y.)* **11**, 458 (1962).
3. SELIGMAN, A. M.: *J. nat. Cancer Inst.* **9**, 13 (1948).
4. BARNES, F. W. JR., DUPFER, H., HENRY, S. S.: *Yale J. Biol. Med.* **24**, 384 (1952).

5. ROBERTS, A. N.: *Amer. J. Path.* **44**, 411 (1964).
6. SMITH, W. W., ALDERMAN, I. M., GILLESPIE, R. E.: *Amer. J. Physiol.* **191**, 124 (1957).
7. SCHRADER, H.: *Amer. J. Path.* **44**, 401 (1964).
8. SCHRADER, W. H., WOOLFREY, B. F., BRUNNING, R. D.: *Amer. J. Path.* **44**, 597 (1964).
9. SKARNES, R. C., ROSEN, F. S.: Host-dependent detoxification of bacterial endotoxin. In: KADIS, S., WEINBAUM, G., AJL, S. J. (eds): *Microbial Toxins. Vol. 5. Bacterial endotoxins*, Academic Press, New York 1971, p. 151.
10. BRAUDE, A. I., CAREY, F. J., SUTHERLAND, D., ZALESKY, M.: *J. clin. Invest.* **34**, 850 (1955).
11. BRAUDE, A. I., CAREY, F. J., ZALESKY, M.: *J. clin. Invest.* **34**, 858 (1955).
12. CAREY, F. J., BRAUDE, A. I., ZALESKY, M.: *J. clin. Invest.* **37**, 441 (1958).
13. DOERING, P., CLEMENS, H., FRITZE, E.: *Z. ges. exp. Med.* **131**, 334 (1959).
14. SMITH, R. T., BRAUDE, A. I., CAREY, F. J.: *J. clin. Invest.* **36**, 695 (1957).
15. NOYES, H. E., MCINTURE, C. R., BLAHUTA, G. J.: *Proc. Soc. exp. Biol. (N. Y.)* **100**, 65 (1959).
16. CHEDID, L., SKARNES, R. C., PARANT, M.: *J. exp. Med.* **117**, 561 (1963).
17. SKARNES, R. D.: *Ann. N. Y. Acad. Sci.* **133**, 644 (1966).
18. ROWLEY, D., HOWARD, J. G., JENKIN, C. R.: *Lancet* **1**, 366 (1956).
19. HOWARD, J. G., ROWLEY, D., WARDLAW, A. C.: *Immunology* **1**, 181 (1958).
20. ROWLEY, D., ALI, W., JENKIN, C. R.: *Brit. J. exp. Path.* **39**, 90 (1958).
21. BERCZI, I., BERTÓK, L., BEREZNAI, T.: *Canad. J. Microbiol.* **12**, 1070 (1966).
22. SONNEN, N.: *Proc. Soc. exp. Biol. (N. Y.)* **134**, 773 (1970).
23. CSÁKÓ, GY., SZILÁGYI, T., CSERNYÁNSZKY, H., GAZDY, E., TÓTH, S.: *Kísér. Orvostud.* **26**, 122 (1974).
24. CSÁKÓ, G., REICHEL, A., CSERNYÁNSZKY, H., REICHEL, U.: *Pflügers Arch. ges. Physiol.* **353**, 151 (1975).
25. DAY, N. K., GOOD, R. A., FINSTAD, J., JOHANNSEN, R., PICKERING, R. J., GEWURZ, H.: *Proc. Soc. exp. Biol. (N. Y.)* **133**, 1397 (1970).
26. KRECKE, M. J., FRITSCH, H., WILLMANN, M., URBASCHEK, R.: *Naturwissenschaften* **7**, 376 (1969).
27. BOIVIN, A., MESROBEANU, L.: *Rev. Immunol.* **1**, 553 (1935).
28. DZVONYÁR, J., RUZICKSA, G., DESEŐ, G., CSABA, B.: *Amer. J. Obstet. Gynec.* **106**, 721 (1970).
29. VON ESCHEN, K. B., RUDBACH, J. A.: *J. infect. Dis.* **129**, 21 (1974).
30. BENACERRAF, B., SEBESTYÉN, M. M.: *Fed. Proc.* **16**, 860 (1957).
31. FRITZER, E., DOERING, P., HERLYN, H.: *Z. ges. exp. Med.* **132**, 321 (1959).
32. JORDAN, M. E.: In: DOWNEY, H. (ed.): *Handbook of Haematology. Vol. 2. Hafner Publications*, New York 1965, p. 771.
33. FOXON, G. E., ROWSON, H.: *Quart. J. microscop. Sci.* **97**, 47 (1956).
34. ANDREW, W.: *Comparative Hematology*. Grune and Stratton, New York 1965, p. 99.
35. CSÁKÓ, G., FEKETE, B., SZEGEDI, G., GERGELY, P.: *Kísér. Orvostud.* **26**, 83 (1974).
36. BERRY, L. J.: Metabolic effects of bacterial endotoxins. In: KADIS, S., WEINBAUM, G., AJL, S. J. (eds): *Bacterial endotoxin. Vol. 5. Academic Press*, New York, 1971, p. 165.
37. ATKINSON, J. P., FRANK, M. M.: *J. clin. Invest.* **53**, 1742 (1974).

Address of the authors:

TIBOR SZILÁGYI, GYÖRGY DESEŐ
Institute of Pathophysiology, University Medical School
H-4012 Debrecen, P.O.B. 23, Hungary

**ABSTRACTS OF
1975 EUROPEAN IMMUNOLOGY MEETING
16TH WORKSHOP "BACTERIAL ENDOTOXINS"**

AMSTERDAM, SEPTEMBER 17-18, 1975

UNDER THE AUSPICES OF THE
INTERNATIONAL UNION OF IMMUNOLOGICAL SOCIETIES

Chairman: L. BERTÓK, Budapest, Hungary
Co-chairman: O. LÜDERITZ, Freiburg, Germany

Preface

When publishing these Abstracts of the "Bacterial Endotoxins" Workshop of the 1975 European Immunology Meeting held in Amsterdam, the Netherlands, we have been glad to see from the great number of official and interested participants from various countries, the valuable contributions delivered at the Workshop and the useful discussions that endotoxin research is in the centre of interest all over the world.

We should like to take this opportunity to express our sincere thanks to the Committee of the European Immunology Meeting, particularly to Professor LANGEVOORT, Secretary General of the Meeting, and his co-workers for the excellent organization and sponsoring this Workshop. Special thanks are due to Mrs. ÁGNES KLIE for valuable technical assistance in the course of preparation of this volume.

September, 1975

O. LÜDERITZ
Freiburg

L. BERTÓK
Budapest

BIOCHEMICAL BASIS OF ENDOTOXIN TOXICITY

M. K. AGARWAL

Institut National de la Santé et de la Recherche Médicale, Paris, France

Whereas specific immunological responses are elicited with a delay of some days, alterations in various biochemical parameters are observed within 1–2 hours after endotoxin administration in a number of animal species. Generally, the levels of several hepatic enzymes such as tryptophan pyrrolase, phosphoenol pyruvate carboxykinase, of metabolites such as glycogen or NAD, and intermediates in the energetic homeostasis are lowered by the toxin, increased by the glucocorticoid hormones and maintained at the normal level when the toxic effects of endotoxin are annihilated by the countereffects of the protective agent (e.g. cortisone). Antagonism is also noted in the synthesis of cellular RNA, DNA and protein. However, no unitary process has as yet been singled out to possessing a cause and effect relationship between toxicity and survival. In the intact animal it is not possible to study whether the toxin exerts a direct effect on the organ, the cell or the process in question, especially since endotoxin is phagocyted by the cells of the reticuloendothelial system (R.E.S.) in the liver and elsewhere and since the biochemical parameters are a property of the organ-specific epithelial cells. Experiments with tissue slices suffer from the problems of permeability and uptake *in vitro* and are not readily reproducible. Isolated cells in culture are theoretically

ideal but exhibit loss of functions and undergo neoplastic type transformation that renders them unsuitable for relevant biochemical studies. Primary explants of hepatocytes and R.E. cells, as well as hybrids between these two cellular types appear more promising. At the moment, isolated liver preparations perfused *in vitro* offer the best solution to investigate intact function and morphology with excellent reproducibility. It is tempting to speculate whether the subsequent immunological response is a reflection of the preceding biochemical process, whether both kinds of events are in some way mediated by the R.E. cells, and it seems that the latter may most easily be studied in the intact liver *in vitro*.

AMYLOID RELATED SERUM PROTEIN IN ENDOTOXIN INDUCED AMYLOIDOSIS

R. F. ANDERS, K. NORDSTOGA, J. B. NATVIG and G. HUSBY

Institute of Immunology and Rheumatology, Oslo, Norway

Protein SAA is a serum protein detected with antisera to the amyloid fibril protein AA. In an investigation of the relationship between protein SAA and protein AA we have measured the serum levels of protein SAA during the development of endotoxin-induced amyloidosis in the mink. The highest levels of protein SAA were seen 24 hr after the first endotoxin injection, prior to the antibody response to the endotoxin. The SAA concentration had returned to relatively low levels prior to the onset of amyloidosis at approximately 4 weeks. Endotoxin induced elevation of protein SAA has also been demonstrated in the mouse and rabbit. Furthermore, humans with Gram-negative septicaemia have markedly elevated SAA levels. The mechanism whereby endotoxin triggers the generation of protein SAA is under investigation.

EFFECT OF ENDOTOXIN DEGRADATION PRODUCTS ON GRANULOPOIESIS OF INBRED MICE

R. N. APTE and H. PLUZNİK

*Department of Life Sciences, Bar-Ilan University,
Ramat-Gan, Israel*

Committed progenitor cells for granulocyte and macrophage differentiation (colony forming cells: CFC) proliferate in soft agar cultures to form colonies of granulocytes and/or macrophages. Colony formation is wholly dependent upon the continuous presence in culture of a colony stimulating factor (CSF) that could be derived from serum. Bacterial endotoxins (ET) are lipopolysaccharides (LPS) made up of two parts different in chemical and physical

properties: a hydrophilic sugar part, the polysaccharide (PS), and a hydrophobic lipid part, the lipid A. ET are potent stimulators of the granulopoietic system. Injection of experimental animals with ET causes an acute rise in tissue and serum CSF followed by a subsequent rise in marrow and spleen CFC levels. It has been studied, which part of LPS, the lipid or PS, is active in stimulating the murine granulopoietic system. Lipid A obtained by acid hydrolysis of the LPS and complexed to bovine serum albumin was shown to be active in generating serum CSF and in increasing splenic CFC, although it was less active than the parent LPS, probably because of insufficient solubility or degradation of the lipid A by acid hydrolysis during the isolation procedure. The LPS showed no significant activity at the concentrations used, LPS (glycolipids) from R mutants from *Salmonella minnesota* deficient in different parts of the PS, but containing all the lipid A, were active to the same extent as the complete LPS. The fact that even the most defective LPS from the R mutant R595, which contains only lipid A and KDO, is a potent endotoxin, points to lipid A as the active principle of the LPS molecule in stimulating the granulopoietic system. In addition, a genetic experimental system was used to study the genetic control of LPS-induced granulopoietic responses. Two inbred strains of mice differing in their response to ET were used: a high responder strain (C₃H/eB) reacting to ET by an increase in serum CSF levels and by an elevation of splenic CFC levels, and a low responder strain (C₃H/HeJ) failing to show these responses. It was found in cross breeding experiments between these two strains that the ability to generate serum CSF in response to ET is controlled by a single autosomal dominant gene, while the splenic CFC responses to ET are inherited polygenically. The same genetic control mechanism of the granulopoietic responses described above were observed when the glycolipid R595 was used, indicating again that lipid A is the active principle of the LPS molecule stimulating the granulopoietic system.

BACTEROIDES ANTIBODIES IN HUMAN SERA IN POLAND AND IN HOLLAND

I. BECKMANN, F. MEISEL-MIKOLAJCZYK and J. ZUIJDERDIJN

*Department of Microbiology and Immunology, Institute of Biostructure, Medical Academy,
Warsaw, Poland*

Out of six strains (representative of six *Bacteroides* serotypes Beerens) antigens were extracted by the phenol-water method, and formalin treated sheep erythrocytes were coated. Haemagglutination test was performed with about 400 human sera. More than 200 of these originated from blood donors, the rest from surgical and gynaecological patients. Sera from blood donors were negative in about 70% (titre 1:5 or less). About 30% reacted with anti-

gens of different serotypes (titre 1:10 or higher). The sera of patients in 30% were negative (titre 1:5 or less). About 70% of patients' sera were positive (titre 1:10–1:320). In the blood donor sera, antibodies against serotype D were the most frequent while in patient sera, those against serotype C, especially in Holland. Antibodies against serotype E₁ were more frequent in Poland. In Holland, positive reactions against the antigen of serotype E₂ were more frequent.

RETICULOENDOTHELIAL CELLS AS MEDIATORS OF ENDOTOXIN EFFECTS

L. J. BERRY and R. N. MOORE

The University of Texas at Austin, Texas, USA

Research in our laboratory has concentrated on several aspects of the marked antagonisms elicited in experimental animals treated with bacterial endotoxin and glucocorticoid hormones. These antagonisms are apparently of utmost importance in determining the fate of a poisoned animal since depriving an animal of its own endogenous corticosteroid induced stress response by either surgical or chemical adrenalectomy sensitized the animal up to 1000-fold to the lethal effect of endotoxin. Results obtained previously have demonstrated a distinct antagonism between steroid and endotoxin in certain metabolic reactions believed to be important in regulation of mammalian homeostasis. More specifically, endotoxin inhibits hormonal induced synthesis of hepatic tryptophan oxygenase, an enzyme which regulates tryptophan metabolism, and phosphoenol pyruvate carboxykinase (PEPCK), a key enzyme in gluconeogenesis. Based on experimental and inferential evidence, we believe that an injection of glucocorticoid allows the poisoned animal to maintain at least minimal levels of these essential enzymatic activities, and possibly other related enzymes, necessary for maintenance of homeostasis.

Our most recent research efforts have concentrated on the mechanism of inhibition of PEPCK induction by endotoxin. Based on two observations, this effect of endotoxin has been assumed to be mediated rather than direct: (i) very small doses of endotoxin are sufficient to inhibit daily induction of the enzyme; and (ii) endotoxin has not been found to localize in hepatocytes which contain the inducible enzyme. This is further strengthened by a third observation that endotoxin does not inhibit gluconeogenesis in isolated hepatic parenchymal cells. Evidence has been obtained demonstrating that this inhibition of PEPCK induction is a mediated effect and that phagocytic cells of the reticuloendothelial system are involved in the production of this mediator.

Direct evidence for a host produced inhibitor was obtained by utilizing endotoxin tolerant mice. Mice which had received several doses of endotoxin over a period of one week were able to respond normally to an injection of

cortisol, i.e., PEPCK was fully inducible in these animals even when they were injected with endotoxin. Serum taken from mice 4 hr after endotoxin poisoning, however, significantly inhibited enzyme induction in these mice. The inhibition could not be attributed to contaminating endotoxin since the mice were tolerant to doses of endotoxin greater than that present in the serum. Preliminary evidence indicates that the inhibitory component of this serum is fairly heat stable being only partially inactivated by heating at 56 °C for 30 min and is sensitive to treatment with trypsin.

Based on the assumption that leukocytes are the likely source of the inhibitory substance, we previously found and reported that PEPCK induction by cortisol was not inhibited by endotoxin in mice pretreated with anti-macrophage and antilymphocyte serum. These results suggested but did not prove that macrophages were responsible for the production of this mediator. More recently we have found that mice pretreated for 1 hr with 0.2 ml i.v. of a rabbit anti-mouse thymocyte serum (ATS) were also protected from the inhibitory effect of endotoxin. Enzyme induction by cortisol was not inhibited in ATS pretreated animals. PEPCK induction in ATS treated mice, however, could be inhibited by an intravenous dose of 0.2 ml of serum obtained from mice 4 hr after an injection of 100 µg of endotoxin.

These results indicated that pretreatment with this antithymocyte serum inhibits production of the mediating substance in response to endotoxin. These results, however, did not clarify which cell type, lymphocytes or macrophages, were involved in the production of the mediator. In an attempt to determine which cells were involved, the ATS was absorbed at 37 °C for 90 min with mouse peritoneal macrophages. The macrophage absorbed ATS no longer protected PEPCK induction in cortisol treated mice challenged with endotoxin. The antilymphocyte titre of the antiserum, as judged by *in vitro* cytotoxicity assays and peripheral lymphocyte counts in ATS treated mice, remained practically unchanged after macrophage absorption. These results imply that reduced production of the glucocorticoid antagonizing factor in mice pretreated with this preparation of ATS is due to the incapacitating effects of the antiserum on the phagocytic cells of the reticuloendothelial system.

Since macrophages are cells which have intimate contact with endotoxin through phagocytosis, a more specific study of macrophage involvement in this phenomenon was undertaken. Peritoneal macrophages were incubated overnight in Eagle's minimal essential medium supplemented with 5% fetal calf serum and containing 100 µg of endotoxin/ml. After 17 hr incubation, the culture fluid was decanted, centrifuged at 500 g, and 0.5 ml of clear supernatant fluid was injected intraperitoneally into endotoxin tolerant mice. Supernatant fluid from macrophages cultured without endotoxin served as the control. Addition of 50 µg of endotoxin to control fluid had no significant inhibitory effect of PEPCK inducibility by cortisol because the mice were tolerant.

On the other hand, the supernatant fluid from macrophages cultured 17 hr in the presence of endotoxin totally inhibited enzyme induction in similar animals.

These results indicate that macrophages are a source of metabolic impairment caused by an injection of endotoxin. Detection of an inhibitory substance in the culture fluid of macrophages incubated for 17 hr in the presence of endotoxin but not in its absence established that some compound produced by these cells acts as a mediator.

MECHANISM ON THE RESPONSIVENESS OF B-DERIVED LYMPHOCYTES TO LPS

C. BONA

Institut Pasteur, Paris, France

The binding, capping, shedding, internalization and subsequent fate of ^3H -labelled *Salmonella enteritidis* lipopolysaccharide (LPS) have been studied in parallel experiments on mouse thymic lymphocytes (T-derived) and on splenic lymphocytes of mice homozygous for nude mutation (largely bone-marrow derived lymphocytes). In spite of a quite similar binding and capping of ^3H -LPS in both cell types, only bone marrow (B) cells are transformed by this mitogen. Several differences were found between B and T cells which could explain the selectivity of LPS as non-specific mitogen of B-derived lymphocytes. 1. After incubation at time 0 with ^3H -LPS, peak binding was observed at 12 hr in both cell types followed in the case of B-cells with a marked loss of label, a phenomenon which we call unloading. This phenomenon was suppressed by colchicine and vinblastine. 2. At 37 °C, cell-bound LPS could effectively be chased from B-cells either by homologous (*S. enteritidis*) or heterologous (*Escherichia coli*) unlabelled LPS. These results suggest the presence of receptors for LPS in both cell types and also that the T-cell receptors were significantly different in several respects from those of the B-cells. Furthermore, B-cells rapidly internalized the ^3H -LPS, and after 6 hr label was found in both cytoplasm and nucleus, whereas it remained bound at the periphery throughout 48 hr in the case of T-cells. The role of the internalization of LPS in the triggering of intracellular metabolic events leading to blast transformation which culminates in DNA duplication and cell mitosis is discussed. 3. Both cells shed off the LPS bound to the membrane. In the case of LPS shed by nude mice lymphocytes, 30% of heavy LPS (2×10^6 daltons) was converted in a light material (4×10^4 daltons). This light material was mitogenic for C3H/HeJ mice lymphocytes which cannot be stimulated by native LPS or by heavy LPS. These data suggest that the internalization of processed LPS is relevant for the triggering of blast response of murine B-derived lymphocytes.

INTERACTION OF LPS WITH ERYTHROCYTE MEMBRANE
A FREEZE ETCH STUDY

G. DI PAULI and R. DI PAULI

Universität Konstanz, Konstanz, GFR

Human erythrocytes were incubated at 37 °C with alkali-treated LPS. The surface of the red cell membrane was studied by the freeze etch method in the electron microscope. In comparison with the control cells, various changes in the membrane could be observed. The nature of the changes depended on the time of incubation with LPS. In the early period, vesicles and tubes are observed on the membrane surface. These structures disappear after a certain time and LPS seems to be distributed evenly throughout the membrane.

ADJUVANT EFFECT OF RADIODETOXIFIED ENDOTOXIN

E. ELEKES, L. BERTÓK and K. MERÉTEY

"Frédéric Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene, Budapest and National Institute for Rheumatism and Physiotherapy, Budapest, Hungary

The adjuvant activity of preparations detoxified by potassium methylate or gamma-irradiation and of toxic endotoxin was compared in R/AxLE hooded F₁ hybrid rats. The endotoxin was prepared by the phenol-water method from freshly isolated *Escherichia coli* O89 bacteria. Detoxification of endotoxin was carried out by potassium methylate according to NOWOTNY or by 5 Mrad ⁶⁰Co-gamma irradiation in a NORATON apparatus. Effectivity of the preparations was controlled in LD₅₀ tests. The animals were immunized with single doses of sheep red blood cells and 100 µg of various endotoxin preparations were administered simultaneously. The animals were killed five days after the antigen injection. In the spleen, the nucleated cell count, the relative spleen weight, direct and developed antibody-forming cells were determined and the serum haemolysin titres were estimated.

BACTERIAL LIPOPOLYSACCHARIDES AND LUPUS NEPHRITIS

G. J. FOURNIE, S. IZUI, P. H. LAMBERT and J. J. CONTE

Laboratoire d'Immunopathologie Rénale, Centre d'Hémodialyse Périodique, C.H.R. Purpan, Toulouse, France, and WHO Immunopathology Research Unit, Geneva Blood Centre, Hôpital Cantonal, Genève, Switzerland

The role of anti-DNA antibodies in the development of lupus nephritis is well documented, but the formation of these antibodies is still poorly understood. Among the possible triggering factors, we have studied the effect of

bacterial lipopolysaccharide (LPS) in mice. Anti-DNA antibodies were titrated in serum, using radiolabelled DNA in a direct binding test. Anti-SS and anti-DS DNA antibodies were found in various strains of mice after injection of LPS. The response was dose-dependent and varied quantitatively according to the strain of the injected mice. It was not related to the H2 histocompatibility locus nor to the immune response to LPS. The active part of the LPS molecule for this particular effect appeared to be the lipid A fraction. Further studies have shown that (i) large amounts of DNA are released into the circulation a few hours after a single injection of LPS; (ii) and that a subsequent induction of anti-DNA antibodies is followed by the appearance of fine granular IgM and C3 deposits in the renal glomeruli along the capillary walls and in mesangial areas. Deposits of DNA were also found in the same location using a fluorescent technique. Immunoglobulins eluted from the kidneys showed a high DNA-binding activity. These results indicate that a single injection of LPS in mice could induce the local deposition of DNA-anti-DNA complexes in renal glomeruli. LPS released by infectious agents in the sera of SLE patients, may trigger the development of a lupus-type immune complex nephritis.

SPECIFIC IMMUNE RESPONSES OF DIFFERENT ANIMALS TO LIPID A

M. A. FREUDENBERG

Max-Planck-Institut für Immunbiologie, Freiburg, GFR

Lipid A is the part of bacterial lipopolysaccharides responsible for endotoxicity. A number of biological activities including mitogenicity for mouse B lymphocytes, which are exhibited by intact endotoxin, were also shown to reside in lipid A. When suitably exposed, lipid A acts as a powerful immunogen in a number of animals. In the present study the specific immune response to lipid A of animals showing differences in their sensitivity to endotoxin was investigated. In previous studies, immunization with lipid A of rabbits, goats and dogs, animals known to be highly sensitive to endotoxin, showed that a single injection leads to detectable serum anti-lipid A titres several days later. These titres were increased by subsequent injections of lipid A. Recent investigations in mice,¹ which are less sensitive to endotoxin, revealed that these animals too may be stimulated to produce high anti-lipid A serum titres. In mice, however, a single lipid A injection was completely ineffective and detectable anti-lipid A antibodies appeared in the serum only after a second dose administered not earlier than 4 weeks after the first one. This behaviour toward lipid A immunization was common to all mouse strains (NMRI, CBA/H, C3H, and C3H/HeJ) tested so far. The strains differ in sensitivity and their mitogenic response to lipid A in that the strains NMRI, CBA/H and C3H are sensitive

to the lethal effects and mitogenic activity of lipid A, while C3H/HeJ mice are resistant to both. Thus, the mitogenic responses of mice to lipid A are independent of their ability to respond specifically to lipid A as an antigen. Further, the extreme resistance of C3H/HeJ mice does not seem to be related to an inability of these animals to produce specific anti-lipid A antibodies.

INTERACTION BETWEEN RADIODETOXIFIED ENDOTOXIN AND COMPLEMENT SYSTEM

G. FÜST and L. BERTÓK

*National Institute of Haematology and Blood Transfusion, Budapest and "Frédéric Joliot-Curie"
National Research Institute for Radiobiology and Radiohygiene, Budapest, Hungary*

Escherichia coli O89 lipopolysaccharide (LPS) preparation was treated with different doses (5, 10, 15 and 20 Mrad) of gamma-radiation. Various biological activities (lethal effect, decrease of arterial blood pressure in dogs, and interaction with the complement system) of the parent and irradiated preparations were compared. Irradiation of the LPS preparations decreased significantly and in a dose-dependent manner their lethal and blood pressure depressing effects as well as their ability to activate the complement system. In contrast, radiodetoxified LPS preparations fixed the isolated human C1 more strongly than did the parent LPS. The possible connection between the toxicity of endotoxin and the endotoxin induced complement activation is discussed.

BIOLOGICAL ACTIVITIES OF LIPOPOLYSACCHARIDES IN DIFFERENT SALT FORMS

C. GALANOS

Max-Planck-Institut für Immunbiologie, Freiburg, GFR

Lipopolysaccharides may be deionized by electrodialysis whereby they are obtained in free acid form. Subsequent neutralization with different bases leads to define salt forms which show large differences in their state of aggregation as judged by sedimentation coefficient determinations. Two extreme examples are the sodium and triethylamine salt forms, which in the case of *Salmonella abortus-equi* lipopolysaccharide exhibit sedimentation constants of 87.4 S and 9.9 S, respectively. The biological activity of these two salt forms has been compared in lethal toxicity tests in adrenalectomized mice, in pyrogenicity tests in rabbits and also by their ability to interact with complement *in vitro*. It was found that the lethal toxicity of the low molecular weight triethylamine form was about 10 time higher than that of the high molecular

weight sodium form. Comparable results were obtained in pyrogenicity tests where, again, the activity of the triethylamine form was higher than that of the sodium form by a factor of 3. A complete contrast was observed in the ability to interact with complement. Here the sodium form exhibited a high anticomplementary activity, while the triethylamine form was completely inactive. The high anticomplementary activity of the sodium form and its absence in the triethylamine form was common with all S form lipopolysaccharides tested so far. This conversion of lipopolysaccharides into the low molecular weight triethylamine form leads to the loss of anti-complementary activity while the other biological activities persist completely. This is the first time that the biological activity of lipopolysaccharides could be suppressed selectively.

ISOLATION AND CHARACTERIZATION OF A LIPOPOLYSACCHARIDE-BINDING PROTEIN FROM THE OUTER MEMBRANE OF *SALMONELLA MINNESOTA* Re

R. GEYER and O. WESTPHAL

Max-Planck-Institut für Immunbiologie, Freiburg, GFR

The chemical and biological properties of lipopolysaccharides (LPS) from Gram-negative bacteria have been studied extensively in the recent years. Less is, however, known about the other main constituents of the bacterial outer membrane, the phospholipids and proteins. Recently a protein has been isolated from the outer membrane of *Salmonella minnesota* Re mutants (R595). A striking property of this protein is its high affinity to lipopolysaccharides. The protein was extracted from the bacteria with EDTA and purified by ion exchange chromatography (CM-Sephadex) and preparative polyacrylamide gel electrophoresis (pH 9.5). The isolated protein was homogeneous with regard to molecular weight and charge (SDS polyacrylamide gel electrophoresis, isoelectric focussing). It has a minimum molecular weight of 15 000 daltons; its isoelectric point is at pH 10.3. Serum absorption experiment indicated that the protein is located on the surface of the bacterial cell. The specificity of the LPS-protein interaction was studied in a double diffusion system. In this system, LPS is precipitated by the protein, yielding a single, sharp precipitation line. Lipid A turned out to represent the combining site of the molecule. Therefore the LPS-protein interaction is not species-specific. Control experiments with other polyanions like nucleic acids and basic proteins such as cytochrome C and lysozyme demonstrated that the LPS-protein interaction is at least partly due to ionic forces. The question whether the endotoxicity of lipopolysaccharides was influenced by the protein is under investigation.

BACTERIOPHAGE RECEPTOR SITES ON CORE LIPOPOLYSACCHARIDE
OF SHIGELLA FLEXNERI

E. HANNECART-POKORNI, C. GODARD and J. BEUMER

Institut Pasteur du Brabant, Brussels, Belgium

The F6R rough mutants isolated from *Shigella flexneri* F6S, serotype 5b, and the FH mutants derived from other serotypes of *S. flexneri*, were chemotyped by chemical analysis of their LPS. Different stages of LPS core biosynthesis in *S. flexneri* were observed and the existence of a similar core inside the species was thus confirmed. The study allowed to identify the receptor sites of phages FP3, V, C21, P1 kevir, H⁺, T4, T7, T3 and 6SR. A good correlation between the efficiency of phage plating on whole cells and the phage inhibiting capacity of isolated LPS showed that only structures of the lipopolysaccharide are involved in phage fixation. In *S. flexneri*, the chemical locations of the different phage receptors are phage EP3: KDO-lipid A region; phage V: heptose region or glucose; phage C21: glucose-heptose region; phages P1 kevir, H⁺ T4 and T3: glucose; phage T7: galactose-glucose; phage 6SR: complete core structure.

STRUCTURE OF LIPID A BACKBONE IN VARIOUS BACTERIAL GROUPS

S. HASE and E. RIETSCHER

Max-Planck-Institut für Immunbiologie, Freiburg, GFR

The lipid component (lipid A) of bacterial lipopolysaccharides (LPS) represents the endotoxic centre of LPS. Lipid A from *Salmonella* Re mutants contains phosphorylated β -1,6-linked glucosamine disaccharides which carry fatty acids in ester and amide linkages. Glucosamine disaccharides identical in structure have been reported for lipid A from *Pseudomonas* and *Shigella*. We have extended these studies to various species of *Salmonella*, *Escherichia coli* and *Shigella flexneri*. For this purpose an analytical method was worked out. Treatment of LPS with weak alkali and weak acid was followed by reduction and hydrazine treatment. The lipid A backbone was then methylated and analysed by combined gas-liquid chromatography and mass spectrometry. The results obtained show that all lipid A preparations studied contain β -1,6 linked glucosamine disaccharides. The first analyses of *Xanthomonas*, *Rhodopseudomonas* and *Chromobacterium* show that the lipid A moiety has a structure different from that observed in *Enterobacteriaceae*.

THE IMMUNODETERMINANT OF SHIGELLA SONNEI PHASE I

T. KONTROHR

Institute of Microbiology, University Medical School, Pécs, Hungary

The common sugar constituents and oligosaccharides obtained by partial acid hydrolysis of LPS do not represent the immunodeterminant structure of *Shigella sonnei* phase I bacteria, as proved by the absence of the reaction in serological tests. A serologically highly active fraction could be isolated from the hydrolysate of LPS by treatment in 1-6 N HCl at 100 °C for 14-18 hr. The substance isolated remains on the start line in the solvent systems commonly used in sugar and aminosugar chromatography. It forms a smeared spot in ammonium isobutyrate PS-systems. The substance of amphoteric character with an isoelectric pH between 6.7 and 7.0, and 1200-1500 molecular weight seemed to be an artifact originating from the spontaneous repolymerization of the genuine immunodeterminant. The analytical data suggest a 5-amino-uronic acid which is not identical with the serologically inactive 2-amino-hexuronic acid isolated by ROMANOWSKA *et al.*

A UNIQUE TYPE OF LIPID A WITH ALTERED BIOLOGICAL PROPERTIES IN LPS OF SOME GRAM-NEGATIVE PHOTOSYNTHETIC BACTERIA (RHODOSPIRILLACEAE)

H. MAYER, C. GALANOS, J. ROPPEL and J. WECKESSER

Max-Planck-Institut für Immunbiologie, Freiburg and Institut für Biologie II. der Universität, Freiburg, GFR

Two species of the *Rhodospirillaceae* family, namely the two budding bacteria *Rhodopseudomonas viridis* and *Rhodopseudomonas palustris* were found to have in their lipopolysaccharides a hitherto unknown type of lipid A. It is characterized by the replacement of the usual D-glucosamine (ubiquitous in lipid A of *Enterobacteriaceae* and *Rhodospirillaceae*) by its 3-amino derivative, i.e. 2,3-diamino-2,3-dideoxy-D-glucose. Both amino groups of the latter are substituted by β -hydroxy-myristic acid. Ester linked fatty acids are present in small amounts. Antisera against lipid A of *R. viridis* and *R. palustris* are cross-reactive with each other but not with antisera prepared against lipid A of *Salmonella typhi-murium* or *Salmonella minnesota*. In contrast, lipid A antisera against other members of the *Rhodospirillaceae* family, e.g. *Rhodopseudomonas gelatinosa* and *Rhodospirillum tenue*, showed marked cross reactions with *Salmonella* lipid A, indicating a lipid A-type similar to that of *Enterobacteriaceae*. Preliminary data on biological activities showed that the lethal toxicity of *R. gelatinosa* is comparable to that of *Salmonella* while that of *R. viridis* and *R. palustris* is significantly lower.

BIOLOGICAL ACTIVITY OF ENDOTOXINS EXTRACTED OF
BACTEROIDES STRAINS

F. MEISEL-MIKOLAJCZYK

*Department of Microbiology and Immunology,
Institute of Biostructure, Medical Academy, Warsaw, Poland*

Out of six strains representing six serotypes of the genus *Bacteroides*, cell fractions were extracted by the phenol-water method. All these fractions appeared to be active as antigens within limits of their own serotype. Chemical analysis showed that the fractions are composed of polysaccharides and lipides. Chromatography demonstrated the heterogeneity of their structure. The bacterial fractions appeared to be pyrogenic in the rabbit; the temperature peak occurred two to three hours after injection. The Shwartzman reaction (LSR) was positive when the preparatory dose of *Bacteroides* endotoxin was followed by provocation with *Escherichia coli* or *Sphaerophorus necrophorus* endotoxin. The reactivity of rabbits varied. Both the Cetavlon method and ultracentrifugation at 120 000 g for 4 hr failed to achieve purification of endotoxin from the nucleinic compounds. Preliminary experiments indicated that the *Bacteroides* fractions are less toxic for mice than the *Sphaerophorus* fractions. In mice, *Bacillus fragilis* serotype E₁ endotoxin injected intraperitoneally caused a rapid decrease in the peripheral leukocyte count; the lowest count occurred after 90–120 min. During the first 30–60 min a decrease of neutrophils and simultaneously a rise of mononuclears were observed. Transformation of rabbit lymphocytes under the influence of endotoxin was noted.

RELATION OF STRUCTURE TO FUNCTION IN BACTERIAL
ENDOTOXINS

A. NOWOTNY, U. H. BEHLING and H. L. CHANG

*Department of Microbiology and Immunology, School of Medicine, Temple University,
Philadelphia, Pennsylvania, USA*

This is the first report describing *in vivo* biologic activities elicited by a non-toxic, polysaccharide-rich, water soluble fraction obtained by partial acid hydrolysis from endotoxic lipopolysaccharide. The two activities present in this preparation were (a) mouse bone marrow cell colony formation stimulation (CSF), and (b) protection of mice against lethal irradiation. With polysaccharide-deficient rough mutants of *Salmonella minnesota*, CSF-inducing activity could be restricted to the "core" region of the LPS structure. Sixty-minute hydrolysis with 1 N HCl at 100 °C or 0.1 M sodium metaperiodate oxidation at cold room temperature completely abolished the CSF-inducing

activity of the preparation, whereas it showed considerable resistance to mild alkaline hydrolysis. These findings indicate that the active component in the preparation is carbohydrate in nature. Lipid preparations from smooth LPS or from Re rough mutants are either much less active or completely inactive in the above two assays. The fully active polysaccharide-rich preparation was found to be inert in seven other characteristic endotoxicity parameters.

SPECIFICITY OF THE ACTIVE ANTIPYROGENIC TEST

B. RALOVICH, A. VERTÉNYI and Cs. LÁNG

*Institute of Microbiology, University Medical School,
Pécs, Hungary*

In studies of active and passive antiendotoxic as well as of antipyrogenic immunity it was observed that these phenomena were characterized by immunospecificity, as with all immune processes. It was supposed that in spite of the seemingly similar biological effect of the endotoxins of Gram-negative bacteria injected parenterally, the antigenicity of the structure responsible for pyrogenicity in the different bacteria might not be the same in other words their toxic group, lipid A, might differ in chemical structure. Therefore the specificity of active antipyrogenic immunity has been investigated by controlling the usefulness of the active antipyrogenic test in rabbits immunized with *Salmonella minnesota* Re 595, or *Salmonella typhi-murium* SL 1102, or *Escherichia coli* O83 or *E. coli* F 576 vaccine and after immunization the rabbits were tested with homologous or heterologous glycolipid or lipopolysaccharide.

LIPID-A INDUCES THE QUICKEST AND MOST POLYSPECIFIC IMMUNE RESPONSE

W. R. RANK, U. FLÜGGE-RANK, R. GURTNER and
D. BITTER-SUERMAN

*Max-Planck-Institut für Biochemie, Martinsried and Institut für Medizinische Mikrobiologie
der Universität, Mainz, GFR*

Purified lipid A without adjuvant prepared from R 595 bacteria grown in antigen-free synthetic medium induces an early immune response *in vivo* with a peak at day 3 after stimulation preceding the IgM response. The lipid is an immunogen comparable in strength with sheep red cells. A single injection may give rise to 150 000 bactericidal human complement-dependent direct antibody plaque forming cells per mouse spleen. The antibody is directed

against the red cells of several species and against many bacterial strains. It is also directed against a variety of polysaccharide components of lipopolysaccharides which are unrelated to lipid A and which belong to different sero- and chemotypes. This raises the question whether the multiple specificities of the earliest antibodies *in vivo* can be achieved without any relationship to the structure of the inducing immunogen, and whether the final high specificity can be achieved in secondary steps. The dependence on the guinea pig complement component C4 shows that the lipid A induced principle is of immunoglobulin nature and activates the classical complement pathway. The thymus is not necessary for the early reaction since thymectomized and congenitally athymic mice respond well to lipid A.

SUPPRESSION OF ENDOTOXIN-FEVER BY LIPID A ANTISERUM

E. RIETSCHER and O. LÜDERITZ

Max-Planck-Institut für Immunbiologie, Freiburg, GFR

Lipopolysaccharides (LPS, endotoxins) of Gram-negative bacteria consist of a species-specific heteropolysaccharide and a ubiquitous lipid component, termed lipid A. The polysaccharide determines the serological specificity of LPS; lipid A on the other hand determines their endotoxic activity. Thus, lipid A is responsible for many endotoxin effects such as pyrogenicity, lethality, induction of the Schwartzman reaction, etc. In the rabbit, lipid A acts as a powerful immunogen capable of engendering antibodies specific to lipid A. These antibodies cross-react with lipid A from LPS of most Gram-negative bacteria due to structural similarities of the lipid A preparations (GALANOS *et al.*, 1971). It was postulated earlier (WATSON and KIM, 1963), that lipid A specific immunoglobulins could *in vivo* neutralize lipopolysaccharide toxicity. We have tested the possible protective effect of lipid A antiserum using lipid A (and LPS) induced fever in rabbits as a test system. In experiments involving passive serum transfer it was found that lipid A antiserum suppresses lipid A and LPS pyrogenicity, provided that the test animals were pretreated with lipid A or LPS. This shows that lipid A antiserum mediates protection against endotoxin fever, but not in the classical antitoxin-toxin manner. The reason why a preparative endotoxin (lipid A) injection is required is not known. Apparently, besides humoral factors (lipid A antiserum) also other factors, perhaps cellular, participate in this system of endotoxin immunity. The recent finding that lipid A antiserum prevents the local Schwartzman reaction in rabbits indicates that the antiserum may offer protection against a number of endotoxin activities. Its possible protective power is investigated in other systems.

The Journal of General Microbiology

Volume 96, Part I, January 1977

ISSN 0022-2720 and 0022-2738

Published by Cambridge University Press, The Edinburgh Building, Shaftesbury Road, Cambridge CB2 2RU, England, and 32 Avenue of the Americas, New York, NY 10013-2473, USA

INDEX

- Lantos, J.: Transfer of Erythromycin Resistance in *Staphylococcus aureus* 107
- Czirók, É., Milch, H., Madár, J., Semjén, G.: Characterization of *Escherichia coli* Sero-groups Causing Meningitis, Sepsis and Enteritis. I. Serological Properties and Animal Pathogenicity of O18, O78 and O83 Isolates 115
- Milch, H., Czirók, É., Madár, J., Semjén, G.: Characterization of *Escherichia coli* Sero-groups Causing Meningitis, Sepsis and Enteritis. II. Classification of *Escherichia coli* O78 Strains by Phage Sensitivity, Colicin Type and Antibiotic Resistance .. 127
- Financsek, I., Kétyi, I.: Phenotypic Correction of Nonsense Mutation Carrying Non-Converting PE5 Phages in *Shigella flexneri* with Suppressor Gene 139
- Szilágyi, T., Deseő, Gy.: Distribution of ^{32}P -Labelled Endotoxin in the Frog 149
- Abstracts of 1975 European Immunology Meeting. 16th Workshop "Bacterial Endotoxins" 157

CONTENTS

1-2	1-2	1-2	1-2
3-4	3-4	3-4	3-4
5-6	5-6	5-6	5-6
7-8	7-8	7-8	7-8
9-10	9-10	9-10	9-10
11-12	11-12	11-12	11-12
13-14	13-14	13-14	13-14
15-16	15-16	15-16	15-16
17-18	17-18	17-18	17-18
19-20	19-20	19-20	19-20
21-22	21-22	21-22	21-22
23-24	23-24	23-24	23-24
25-26	25-26	25-26	25-26
27-28	27-28	27-28	27-28
29-30	29-30	29-30	29-30
31-32	31-32	31-32	31-32
33-34	33-34	33-34	33-34
35-36	35-36	35-36	35-36
37-38	37-38	37-38	37-38
39-40	39-40	39-40	39-40
41-42	41-42	41-42	41-42
43-44	43-44	43-44	43-44
45-46	45-46	45-46	45-46
47-48	47-48	47-48	47-48
49-50	49-50	49-50	49-50
51-52	51-52	51-52	51-52
53-54	53-54	53-54	53-54
55-56	55-56	55-56	55-56
57-58	57-58	57-58	57-58
59-60	59-60	59-60	59-60
61-62	61-62	61-62	61-62
63-64	63-64	63-64	63-64
65-66	65-66	65-66	65-66
67-68	67-68	67-68	67-68
69-70	69-70	69-70	69-70
71-72	71-72	71-72	71-72
73-74	73-74	73-74	73-74
75-76	75-76	75-76	75-76
77-78	77-78	77-78	77-78
79-80	79-80	79-80	79-80
81-82	81-82	81-82	81-82
83-84	83-84	83-84	83-84
85-86	85-86	85-86	85-86
87-88	87-88	87-88	87-88
89-90	89-90	89-90	89-90
91-92	91-92	91-92	91-92
93-94	93-94	93-94	93-94
95-96	95-96	95-96	95-96
97-98	97-98	97-98	97-98
99-100	99-100	99-100	99-100

Printed in Hungary

The Journal of General Microbiology

Volume 98, Part I, January 1977

DEVELOPMENT AND STRUCTURE

Excystment of Axenically Prepared Cysts of *Hartmannella culbertsoni*. By D. C. KAUSHAL and O. P. SHUKLA

The Influence of Sterols on Meiosis in *Phytophthora cactorum*. By C. G. ELLIOTT and E. SANSOME

Structure of Mitochondria and Vacuoles of *Candida utilis* and *Schizosaccharomyces pombe* Studied by Electron Microscopy of Serial Thin Sections and Model Building. By M. T. DAVISON and P. B. GARLAND

Locus of Blue and Near Ultraviolet Reversible Photoreaction in the Stages of Conidial Development in *Botrytis cinerea*. By Y. SUZUKI, T. KUMAGAI and Y. ODA

PHYSIOLOGY AND GROWTH

The Effect Anaerobiosis and Bile Salts on the Growth and Toxin Production by *Vibrio cholerae*. By P. B. FERNANDES and H. L. SMITH. JR

Growth of *Spirillum lipoferum* at Constant Partial Pressures of Oxygen, and the Properties of its Nitrogenase in Cell-free Extracts. By Y. OKON, J. P. HOUCHEINS, S. L. ALBRECHT and R. H. BURRIS

The Effect of Nalidixic Acid on the Cell Cycle of Synchronous *Rhodospseudomonas palustris* Cultures. By D. WESTMACOTT and S. B. PRIMROSE

Fluctuations in Buoyant Density during the Cell Cycle of *Escherichia coli* K12: Significance for the Preparation of Synchronous Cultures by Age Selection. By R. K. POOLE

BIOCHEMISTRY

Mechanism of Compact-colony Formation by Strains of *Staphylococcus aureus* in Serum Soft Agar. By K. YOSHIDA, T. OHTOMO and Y. MINEGISHI

The Metabolism of Starch, Glucose, Amino Acids, Purines, Pyrimidines and Bacteria by the Rumen Ciliate *Polyplastron multivesiculatum*. By G. S. COLEMAN and J. I. LAURIE

Adenylate Energy Charge during Batch Culture of *Benetkea natriegens*. By D. F. NIVEN, P. A. COLLINS and C. J. KNOWLES

Solid Media Containing Carboxymethylcellulose to Detect Cx Cellulase Activity of Micro-organisms. By L. HANKIN and S. L. ANAGNOSTAKIS

Microbial Metabolism of Amino Alcohols. Control of Formation and Stability of Partially Purified Ethanolamine Ammonia-lyase in *Escherichia coli*. By C. M. BLACKWELL, F. A. SCARLETT and J. M. TURNER

Synthesis of Ribosomal and Transfer Ribonucleic Acids in Yeast During a Nutritional Shift-up. By C. WALDRON

An Alginate Lyase from *Azotobacter vinelandii* Phage. By I. W. DAVIDSON, C. J. LAWSON, and I. W. SUTHERLAND

Aerobic and Anaerobic Bacterial Respiration Monitored by Electrodes. By P. JOHN.

GENETICS AND MOLECULAR BIOLOGY

The Nature of the Proteins in 'Chloramphenicol Particles' from *Escherichia coli* A19 (Hfr *rel met rns*). By J. SYKES, E. METCALF and J. D. PICKERING

The Nature of the Proteins Present in the 'Relaxed Particles' from Methionine-starved *Escherichia coli* A19 (Hfr *rel met rns*). By J. SYKES, E. METCALE and J. D. PICKERING

Plasmid Modification of Radiation and Chemical-mutagen Sensitivity in *Pseudomonas aeruginosa*. By P. LEHRBACH, A. H. C. KUNG, B. T. O. LEE and G. A. JACOBY

Construction and Properties of Hybrids Obtained in Interspecific Crosses between *Streptomyces coelicolor* A3(2) and *Streptomyces griseus* Kr. 15. By N. D. LOMOVSKAYA, T. A. VOEYKOVA and N. M. MKRTUMIAN

Genetic Determination of Methylenomycin Synthesis by the SCP1 Plasmid of *Streptomyces coelicolor* A3(2). By R. KIRBY and D. A. HOPWOOD

Introduction of Bacteriophage Mu into *Pseudomonas solanacearum* and *Rhizobium meliloti* using the R Factor RP4. By C. BOUCHER, B. BERGERON, M. BARATE DE BERTALMIO and J. DÉNARIÉ

Genetic Transformation in *Methylobacterium organophilum*. By M. O'CONNOR, A. WOPAT and R. S. HANSON

MEDICAL MICROBIOLOGY

Variation in the Prevalence of Antibiotic Resistance of *Staphylococcus aureus* from Human Skin and Nares. By W. C. NOBLE

TAXONOMY

Taxonomy of the Genus *Serratia*. By P. A. D. GRIMONT, H. L. C. DULONG DE ROSNAY and P. H. A. SNEATH

Fatty and Mycolic Acid Composition of *Bacterionema matruchotii* and Related Organisms. By L. ALSHAMAONY, M. GOODFELLOW, D. E. MINNIKIN, G. H. BOWDEN and J. M. HARDIE

SHORT COMMUNICATIONS

Prototrophic Growth of *Citrobacter freundii* and the Biochemical Basis for its Apparent Growth Requirements in Aerated Media. By C. W. KEEVIL, J. S. HOUGH and J. A. COLE

A Study of Genetic Transformation in *Gloeocapsa alpicola*. By C. I. DEVILLY and J. A. HOUGHTON

Separation of Glutamate Dehydrogenases of *Coprinus cinereus* on Polyacrylamide Gels. By M. O. FAWOLE

Measurement of Growth Rates of Streptomycetes: Comparison of Turbidimetric and Gravimetric Techniques. By T. H. FLOWERS and S. T. WILLIAMS

Some Features of Mannitol Metabolism in *Rhizobium japonicum*. By L. D. KUYKENDALL and G. H. ELKAN

Bacterial Surfaces as Revealed by the High Resolution Scanning Electron Microscope. By K. AMAKO and A. UMEDA

Distribution of Plasma-membrane Fragments during Zonal Centrifugations of Homogenates from Aerobic *Saccharomyces cerevisiae*. By T. NURMINEN, L. TASKINEN and H. SUOMALAINEN

Wall Structure of the Sporonts of *Encephalitozoon cuniculi* Grown in Human Fibroblasts. By R. C. HAMILTON, J. C. COX and D. PYE

Energy Conservation in *Thiobacillus neapolitanus* C: Sulphide and Sulphite Oxidation. By J. W. DROZD

Volumes 98—103 (1977) £15.50 (\$42.00 U.S.A. and Canada) each. Single parts £10.00 (\$26.00 U.S.A. and Canada) each.

Enquiries about advertising in the journal should be sent to the Press.

CAMBRIDGE UNIVERSITY PRESS

Bentley House, 200 Euston Road, London NW1 2DB
32 East 57th Street, New York, N.Y. 10022.

Die *Acta Microbiologica* veröffentlichen Abhandlungen aus dem Bereiche der Mikrobiologie in deutscher, englischer, französischer und russischer Sprache.

Die *Acta Microbiologica* erscheinen in Heften wechselnden Umfanges. Mehrere Hefte bilden einen Band.

Die zur Veröffentlichung bestimmten Manuskripte sind an folgende Adresse zu senden:
Acta Microbiologica, Staatliches Institut für Hygiene, H-1966 Budapest, P. O. B. 64, Ungarn

An die gleiche Anschrift ist auch jede für die Redaktion bestimmte Korrespondenz zu richten. Abonnementspreis pro Band: \$ 32.00.

Bestellbar bei dem Außenhandels-Unternehmen «*Kultúra*» (H-1389 Budapest 62, P. O. B. 149, Bankkonto Nr. 218-10990) oder bei seinen Auslandsvertretungen und Kommissionären.

Les *Acta Microbiologica* paraissent en français, allemand, anglais et russe et publient des travaux du domaine de la microbiologie.

Les *Acta Microbiologica* sont publiés sous forme des fascicules qui seront réunis en volumes.

On est prié d'envoyer les manuscrits destinés à la rédaction à l'adresse suivante:

Acta Microbiologica, Institut National d'Hygiène Publique, H-1966 Budapest, P. O. B. 64, Hongrie

Toute correspondance avec la rédaction doit être envoyée à cette même adresse.

Le prix de l'abonnement est de \$ 32.00 par volume.

On peut s'abonner à l'Entreprise pour le Commerce Extérieur «*Kultúra*» (H-1389 Budapest 62, P. O. B. 149, Compte courant No. 218-10990) ou à l'étranger chez tous les représentants ou dépositaires.

«*Acta Microbiologica*» публикуют трактаты из области микробиологии на русском, немецком, английском и французском языках.

«*Acta Microbiologica*» выходят отдельными выпусками разного объема. Несколько выпусков составляют один том.

Предназначенные для публикации рукописи следует направлять по адресу:

Acta Microbiologica, Институт Здравоохранения, H-1966 Budapest, P. O. B. 64, Венгрия

По этому же адресу направлять всякую корреспонденцию для редакции.

Подписания цена — \$ 32.00 за том.

Заказы принимает предприятие по внешней торговле «*Kultúra*» (H-1389 Budapest 62, P. O. B. 149, Текущий счет № 218-10990), или его заграничные представительства и уполномоченные.

Reviews of the Hungarian Academy of Sciences are obtainable
at the following addresses:

AUSTRALIA

C.B.D. LIBRARY AND SUBSCRIPTION SERVICE,
Box 4886, G.P.O., Sydney N.S.W. 2001
COSMOS BOOKSHOP, 135 Ackland Street, St.
Kilda (Melbourne), Aictoria 3182

AUSTRIA

GLOBUS, Höchstädtplatz 3, 1200 Wien XX

BELGIUM

OFFICE INTERNATIONAL DE LIBRAIRIE, 30
Avenue Marnix, 1050 Bruxelles
LIBRAIRIE DU MONDE ENTIER, 162 Rue du
Midi, 1000 Bruxelles

BULGARIA

HEMUS, Bulvar Ruszki 6, Sofia

CANADA

PANNONIA BOOKS, P.O. Box 1017, Postal Sta-
tion "B", Toronto, Ontario M5T 2T8

CHINA

CNPICOR, Periodical Department, P.O. Box 50,
Peking

CZECHOSLOVAKIA

MAD'ARSKÁ KULTURA, Národní třída 22,
115 66 Praha
PNS DOVOZ TISKU, Vinohradská 46, Praha 2
PNS DOVOZ TLAČE, Bratislava 2

DENMARK

EJNAR MUNKSGAARD, Norregade 6, 1165
Copenhagen

FINLAND

AKATEEMINEN KIRJAKAUPPA, P.O. Box 128,
SF-00101 Helsinki 10

FRANCE

EUROPERIODIQUES S. A., 31 Avenue de Ver-
sailles, 78170 La Celle St. Cloud
LIBRAIRIE LAVOISIER, 11 rue Lavoisier, 75008
Paris
OFFICE INTERNATIONAL DE DOCUMENTA-
TION ET LIBRAIRIE, 38 rue Gay-Lussac, 75240
Paris Cedex 05

GERMAN DEMOCRATIC REPUBLIC

HAUS DER UNGARISCHEN KULTUR, Karl-
Liebknecht-Strasse 9, DDR-102 Berlin
DEUTSCHE POST ZEITUNGSVERTRIEBSAMT,
Strasse der Pariser Kommune 3-4, DDR-104 Berlin

GERMAN FEDERAL REPUBLIC

KUNST UND WISSEN ERICH BIEBER, Postfach
46, 7000 Stuttgart 1

GREAT BRITAIN

BLACKWELL'S PERIODICALS DIVISION, Hythe
Bridge Street, Oxford OX1 2ET
BUMPUS, HALDANE AND MAXWELL LTD.,
Cowper Works, Olney, Bucks MK46 4BN
COLLET'S HOLDINGS LTD., Denington Estate,
Wellingborough, Northants NN8 2QT
WM. DAWSON AND SONS LTD., Cannon House,
Folkestone, Kent CT19 5EE
H. K. LEWIS AND CO., 146 Gower Street, London
WC1E 6BS

GREECE

KOSTARAKIS BROTHERS, International Book-
sellers, 2 Hippokratous Street, Athens-143

HOLLAND

MEULENHOF-BRUNA B.V., Beulingstraat 2,
Amsterdam
MARTINUS NIJHOFF B.V., Lange Voorhout
—11, Den Haag

SWETS SUBSCRIPTION SERVICE, 347b Heere-
weg, Lisse

INDIA

ALLIED PUBLISHING PRIVATE LTD., 13/14
Asaf Ali Road, New Delhi 110001
150 B-6 Mount Road, Madras 600002
INTERNATIONAL BOOK HOUSE PVT. LTD.,
Madame Cama Road, Bombay 400039
THE STATE TRADING CORPORATION OF
INDIA LTS., Books Import Division, Chandralok,
36 Janpath, New Delhi 110001

ITALY

EUGENIO CARLUCCI, P.O. Box 252, 70100 Bari
INTERSCIENTIA, Via Mazzè 28, 10149 Torino
LIBRERIA COMMISSIONARIA SANSONI, Via
Lamarmora 45, 50121 Firenze
SANTO VANASIA, Via M. Macchi 58, 20124
Milano
D. E. A., Via Lima 28, 00198 Roma

JAPAN

KINOKUNIYA BOOK-STORE CO. LTD., 17-7
Shinjuku-ku 3 chome, Shinjuku-ku, Tokyo 160-91
MARUZEN COMPANY LTD., Book Department,
P.O. Box 5056 Tokyo International, Tokyo 100-31
NAUKA LTD., IMPORT DEPARTMENT, 2-30-19
Minami Ikebukuro, Toshima-ku, Tokyo 171

KOREA

CHULPANMUL, Phenjan

NORWAY

TANUM-CAMMERMEYER, Karl Johansgatan
41-43, 1000 Oslo

POLAND

WĘGIERSKI INSTYTUT KULTURY, Marszał-
kowska 80, Warszawa
CKP I W ul. Towarowa 28 00-958 Warsaw

ROUMANIA

D. E. P., București
ROMLIBRI, Str. Biserica Amzei 7, București

SOVIET UNION

SOJUZPETCHATJ — IMPORT, Moscow
and the post offices in each town
MEZHDUNARODNAYA KNIGA, Moscow G-200

SPAIN

DIAZ DE SANTOS, Lagasca 95, Madrid 3

SWEDEN

ALMQVIST AND WIKSELL, Gamla Brogatan 26,
101 20 Stockholm
GUMPERTS UNIVERSITETSBOKHANDEL AB,
Box 346, 401 25 Göteborg 1

SWITZERLAND

KARGER LIBRI AG, Petersgraben 41, 4011 Basel

USA

EBSCO SUBSCRIPTION SERVICES, P.O. Box
1943, Birmingham, Alabama 35201
F. W. FAXON COMPANY, INC., 15 Southwest
Park, Westwood, Mass. 02090
THE MOORE-COTTRELL SUBSCRIPTION
AGENCIES, North Cohocton, N. Y. 14868
READ-MORE PUBLICATIONS, INC., 140 Cedar
Street, New York, N. Y. 10006
STECHELT-MACMILLAN, INC., 7250 Westfield
Avenue, Pennsauken N. J. 08110

VIETNAM

XUNHASABA, 32, Hai Ba Trung, Hanoi

YUGOSLAVIA

JUGOSLAVENSKA KNJIGA, Terazije 27, Beograd
FORUM, Vojvode Mišića 1, 21000 Novi Sad

ACTA MICROBIOLOGICA

ACADEMIAE SCIENTIARUM
HUNGARICAE

ADIUUVANTIBUS

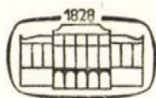
L. ALFÖLDI, I. BÉLÁDI, I. DÖMÖK, E. FARKAS, G. FARKAS,
L. FERENCZY, I. FÖLDES, I. HORVÁTH, I. JOÓ, I. KÉTYI,
L. KESZTYŰS, B. LÁNYI, I. NÁSZ, I. M. SZABÓ, L. VÁCZI,
J. WEISZFEILER

REDIGIT

G. IVÁNOVICS

TOMUS XXIV

FASCICULUS 3



AKADÉMIAI KIADÓ, BUDAPEST

1977

AMAH 5 24(3) 175-262 1977

Acta microbiol. Acad. Sci. hung.

ACTA MICROBIOLOGICA

A MAGYAR TUDOMÁNYOS AKADÉMIA
MIKROBIOLÓGIAI KÖZLEMÉNYEI

KIADÓHIVATAL: 1054 BUDAPEST, ALKOTMÁNY UTCA 21.

A szerkesztőbizottság elnöke:
IVÁNOVICS GYÖRGY
akadémikus

Főszerkesztő:
LÁNYI BÉLA

Az *Acta Microbiologica* német, angol, francia és orosz nyelven közöl értekezéseket a mikrobiológia tárgyköréből.

Az *Acta Microbiologica* változó terjedelmű füzetekben jelenik meg. Több füzet alkot egy kötetet.

A közlésre szánt kéziratok a következő címre küldendők:

Acta Microbiologica, Országos Közegészségügyi Intézet, 1966 Budapest, Pf. 64.

Ugyanerre a címre küldendő minden szerkesztőségi levelezés.

Megrendelhető a belföld számára az Akadémiai Kiadónál (1363 Budapest Pf. 24. Bankszámla 215-11448), a külföld számára pedig a „Kultúra” Külkereskedelmi Vállalatnál (1389 Budapest 62, Pf. 149, bankszámla: 218-10990) vagy annak külföldi képviselőinél és bizományosainál.

The *Acta Microbiologica* publish papers on microbiological subjects in English, German, French and Russian.

The *Acta Microbiologica* appear in parts of varying size, making up volumes.

Manuscripts should be addressed to

Acta Microbiologica, National Institute of Hygiene, H-1966 Budapest, P.O.B. 64, Hungary

Correspondence with the editors should be sent to the same address.

The rate of subscription is \$ 32.00 a volume.

Orders may be placed with “Kultúra” Foreign Trade Company (H-1389 Budapest 62, P.O.B. 149, Account No. 218-10990) or with representatives abroad.

QUANTITATION OF SEVEN ELEMENTS IN MYCOBACTERIUM PHLEI AND MYCOBACTERIUM BOVIS BY NEUTRON ACTIVATION ANALYSIS

VALENTINA KARASSEVA, ILONA SZIKLAI and MARIA ÖRDÖGH

*Microbiological Research Group and Central Research Institute for Physics,
Hungarian Academy of Sciences, Budapest*

(Received May 7, 1976)

Quantitative determination of the elements potassium, sodium, manganese, magnesium, iron, cobalt and zinc was performed in mycobacteria by neutron activation analysis. *Mycobacterium phlei* ATCC 19 249 at different phase of growth (4, 8, 13, 23 and 37 days old cultures), and 14 days old *Mycobacterium bovis* BCG cultures and uninoculated semi-synthetic Sauton culture media were examined. The elements studied could be divided into three groups; sodium, potassium and magnesium could be regarded as major, iron as minor, and zinc, manganese and cobalt as trace elements. *M. phlei* contained, with the exception of zinc, higher amounts of elements than *M. bovis*. Other metals (aluminium, antimony, rubidium) could also be detected.

Data are scarce concerning the various elements in microorganisms [1–10], and conventional analytical procedures do not allow to measure trace elements. The application of neutron activation analysis combined with subsequent γ -spectrometry has made it possible to determine a number of trace elements with high sensitivity and great accuracy [11].

The aim of the present study was to investigate the changes in the concentrations of some elements in *Mycobacterium phlei* cells of different age, and in *Mycobacterium bovis* strain BCG grown on semisynthetic medium.

Few reports have dealt with the subject [6, 12, 13] and no such study has been made of the genus *Mycobacterium*. Since earlier data show considerable differences, it seemed necessary to determine some elements of *M. phlei* and BCG by a modern, reliable method. It was not our aim to study the “iron uptake reaction” [14].

Materials and methods

Microorganisms and growth medium. The mycobacterial strains used in this study were *M. phlei* ATCC 19 249 and *M. bovis*, strain BCG P1102. They were grown on Sauton liquid medium slightly modified by us. The medium contained 0.4% L-asparagine, 0.2% citric acid, 0.05% bipotassium hydrophosphate, 0.05% magnesium sulphate, 0.005% ferric ammonium citrate and 1% D-glucose (instead of 6% glycerol used by Sauton) in demineralized water; the final pH was adjusted to 7.2. Glucose was treated separately and added to the medium aseptically. Culture media were distributed in 500 ml amounts into 2 litre Roux flasks.

Growth conditions and cell harvesting. Stock cultures were maintained on Löwenstein-Jensen slants. Sauton's media were inoculated with heavy suspensions of organisms from the surfaces of 3–5 day old cultures of *M. phlei* and of 7–9 day old cultures of BCG. The cells were incubated at 37°C and after the required period the actively growing pellicles were harvested, suspended in a known volume of the same medium and transferred into 2 litre Roux flasks

containing 500 ml of Sauton's medium. When the cultures had reached the desired stage of growth, the cells were harvested by sedimenting in a refrigerated centrifuge.

Preparation of samples. In all phases of the study, precautions were taken to reduce the risk of metallic contamination of the samples. The culture glassware was cleaned overnight in strong chromic acid, rinsed four times with tap water and twice with demineralized water, then sterilized by dry heat at 160°C for 1 hr.

The harvested bacteria were washed thoroughly in cold demineralized water in polypropylene centrifuge tubes. After final centrifugation the bacteria were removed and dried to constant weight at 105°C under controlled conditions for determination of the total dry weight, after which they were powdered.

Uninoculated media were also analysed to determine the initial contents of the elements. These samples were evaporated to dryness in a Rotatest glass distiller. After the samples were dried to constant weight, they were powdered and sealed.

Cultures of *M. phlei* harvested after 4, 8, 12, 23 and 37 days of growth and cells of BCG after two weeks of growth were prepared for analysis.

Sample irradiation. The samples and the appropriate standards were irradiated simultaneously in a WWR-S type reactor. The elements were divided into two groups with respect to their nuclear properties. Short irradiations of one or two minutes were applied to determine magnesium, manganese, sodium, and potassium; these were performed with a pneumatic transfer system built into the core of the reactor, at a neutron flux of 2.47×10^{13} n/cm²s. Dried samples in amounts of 15–20 mg were irradiated.

Irradiation lasting for 50 hr were used for iron, cobalt and zinc in a vertical channel of the reactor at a neutron flux of 3×10^{13} n/cm²s. Dried samples of 0.08–0.12 g were used for long irradiations.

At the 300–400°C temperature in the vertical channels, the ampoules containing biological samples may be decomposed during irradiation, therefore prior to irradiation the samples were carbonized at 200°C for a period of 5–6 hr.

After suitable cooling the samples irradiated for a long period were dissolved from the ampoules by nitric acid then destroyed by a mixture of 1:1 concentrated nitric acid and hydrogen peroxide. The nitric acid was removed from the destroyed samples by boiling with hydrogen peroxide and finally the samples were evaporated to dryness.

After short irradiations prior to counting, the samples were allowed to cool for 5–10 min. After irradiations of 50 hr a 12–15 days cooling period was necessary for the decay of the inconveniently high activities arising from the major components (²⁴Na, ⁴²K, etc.).

The gamma spectra of the activated samples were measured by a Nuclear Diodes (Ge)Li detector of 45 cm³ volume, coupled to an NTA-type 1024 channel analyser.

The elements were identified by the nuclear properties of the radioisotopes produced in the nuclear reactions summarized in Table I.

Table I
*Nuclear data of the elements investigated**

Element	Isotope	Nuclear reaction	Gamma energy E (MeV)	Half-life T 1/2
Na	²⁴ Na	²³ Na/n, γ / ²⁴ Na	1.368	15.0 hr
Mg	²⁷ Mg	²⁶ Mg/n, γ / ²⁷ Mg	0.844	9.45 min
K	⁴² K	⁴¹ K/n, γ / ⁴² K	1.524	12.52 hr
Mn	⁵⁶ Mn	⁵⁵ Mn/n, γ / ⁵⁶ Mn	0.846	2.58 hr
Co	⁶⁰ Co	⁵⁹ Co/n, γ / ⁶⁰ Co	1.173**	5.24 yr
			1.332	
Fe	⁵⁹ Fe	⁵⁸ Fe/n, γ / ⁵⁹ Fe	1.098**	45.1 day
			1.291	
Zn	⁶⁵ Zn	⁶⁴ Zn/n, γ / ⁶⁵ Zn	1.115	245 day

* Nuclear data according to CROUTHAMEL et al. [11]

** ⁶⁰Co and ⁵⁹Fe have two characteristic photopeaks

Results and discussion

All cultures formed pellicles on the surface of the medium. Figure 1 shows the yields of *M. phlei* (g dry wt of cells per litre culture) harvested at different intervals. Maximum growth was observed on the fourth day after which the yields diminished, presumably in consequence of autolysis.

A typical gamma spectrum of *M. phlei* after a short irradiation time is shown in Fig. 2, and that after a long irradiation time in Fig. 3.

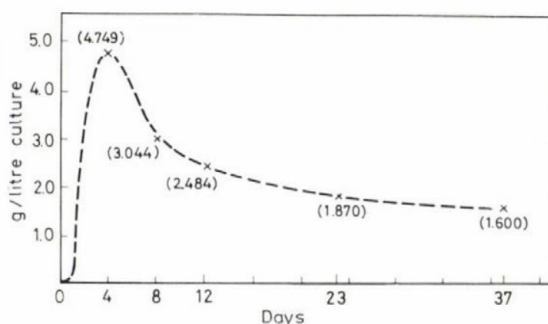


Fig. 1. Yields of *M. phlei*

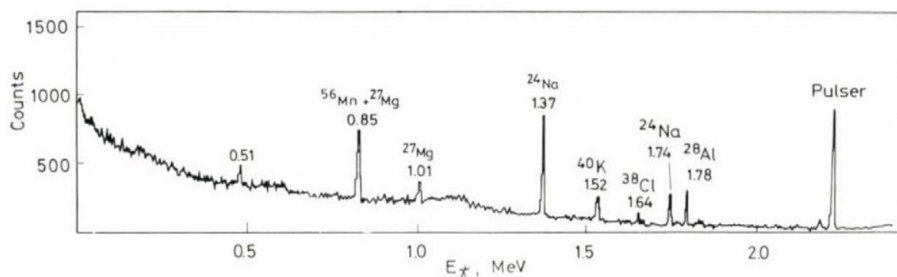


Fig. 2. Typical gamma-spectrum of *M. phlei* after short irradiation

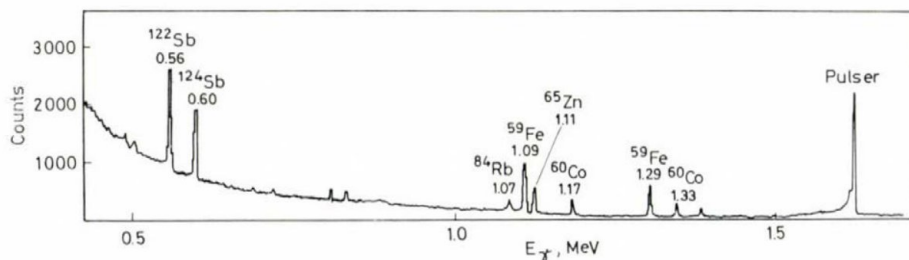


Fig. 3. Typical gamma-spectrum of *M. phlei* after long irradiation

The amounts of sodium, potassium, magnesium, iron, zinc, manganese and cobalt in the organisms are shown in Table II. They could be divided into three groups, i.e. major, minor and trace elements. Sodium, potassium and magnesium may be considered major elements, since they are present in high proportions. Iron may be regarded as minor, while zinc, manganese and cobalt as trace elements.

The contents of the bacteria varied with the age of the culture. Potassium, magnesium and sodium contents of *M. phlei* were highest on the 4th day of growth. In BCG cells harvested on the 14th day, the levels were considerably lower than in *M. phlei*. The peak accumulation of cobalt and manganese in *M. phlei* occurred in 23-day-old cultures.

Minimal changes in the levels of iron could be observed in 4–23-day-old cultures of *M. phlei* with a slight decrease on the 37th day.

Cobalt is taken up by many bacteria [15] but its amount is usually too small to be detected by conventional analytical procedures. By the use of neutron activation analysis it was possible to measure the accumulation of cobalt as well as of manganese and to establish the time of peak accumulation. It seemed that incorporation of manganese took place at the beginning of the phase of declining growth.

Our results concerning the amounts of potassium, magnesium and iron are in good agreement with data reported by DAMBOVICEANU *et al.* [2].

The culture media were also analysed quantitatively (Table II). Three different charges of uninoculated media were prepared and evaporated to dryness. Growth media yielded 10.02 g per litre of dry substance. The media were contaminated by manganese, zinc, cobalt and sodium (these ions were not added to the media). The presence of these elements can be explained by impurities in the ingredients [16–18] and in glassware [19]; chemicals are also often contaminated with as much as 0.05% Mg^{2+} and Ca^{2+} [20], and these elements have been detected in the distilled water used in the preparation of media [13]. This explains that in the mycobacteria used other metal ions: aluminium, antimony and rubidium were also observed (Figs 2 and 3). It was not our aim to quantitate these elements.

Thus, the results showed accumulation by mycobacteria of several elements and that their amounts are higher in *M. phlei* than in BCG, with the exception of zinc, which is more abundant in BCG bacteria. It is concluded that age-dependent changes in the composition of microorganisms have an important role in their biological processes.

Acknowledgements. We are indebted to Professors J. G. WEISZFEILER and I. FÖLDES for valuable help and suggestions during propagation of the manuscript, and to Mr. J. HAJDU and Miss E. VARGA for skilful technical assistance.

Table II

*Amounts of elements in cells of bacteria and in uninoculated culture medium (mg/g dry wt.)**

Elements	Culture medium	<i>M. phlei</i>					<i>M. bovis</i> BCG
		4 days	8 days	12 days	23 days	17 days	14 days
Na	0.440 ± 0.24	1.42	0.145 ± 0.036	0.496 ± 0.017	0.14 ± 0.06	1.12 ± 0.050	0.633 (2)
K	5.53 ± 0.44	9.76 (2)	5.73 ± 0.18	2.86 ± 0.18	2.34 ± 0.17	4.10 ± 0.67	8.86 (2)
Mg	2.41 ± 0.14	2.41 ± 0.14	1.21 ± 0.08	1.03 ± 0.09	1.18 ± 0.15	2.48 ± 0.16	2.36 (2)
Fe	0.93 ± 0.038	0.607 ± 0.146	0.607 ± 0.146	0.590 ± 0.027	0.651 ± 0.043	0.404 ± 0.033	0.211 ± 0.015
Zn	0.038 ± 0.02	0.017 ± 0.0009	0.051 ± 0.0005	0.031 (2)	0.038 ± 0.0013	0.054 ± 0.02	0.046 (2)
Mn	0.0068 ± 0.003	0.0055 ± 0.0003	0.0054 ± 0.0006	0.0045 ± 0.0002	0.0111 ± 0.0007	0.0115 ± 0.0005	0.0017 (2)
Co	0.00018 (3)	0.00038 ± 0.0002	0.00029 ± 0.00002	0.0006 ± 0.00007	0.0026 ± 0.00009	0.00014 ± 0.00002	0.0003 ± 0.00004

* Means $\pm$ sd of individual measurements, except in the cases where the figures in brackets indicate the number of estimations

REFERENCES

1. CURRAN, H. R., BRYNSTETTER, B. C., MYERS, A. T.: *J. Bact.* **45**, 485 (1943).
2. DAMBOVICEANU, A., DORIN, R. 1936. cit. by ASSELINEAU, J.: In MEISSNER, G., SCHMIEDEL, A. (eds): *Mycobacterien und mycobakterielle Krankheiten*. Gustav Fischer Verlag, Jena. Part 2. pp. 107-169.
3. DARZINS, E.: In DARZINS, E. (ed.): *The Bacteriology of Tuberculosis*. University of Minnesota Press, Minneapolis, Minn. 1958. pp. 160-168.
4. DREA, W. F., ANDREJEW, A. cit. by DREA, W. F., ANDREJEW, A. (eds): *The Metabolism of the Tubercle Bacillus*. Charles C. Thomas, Springfield, Ill. 1953.
5. JOHNSON, T. B., BROWN, E. B.: cit. by Porter [10].
6. KRUEGER, W. B., CAREY, W. E., KOLODZELJ, B. J.: *Appl. Microbiol.* **20**, 946 (1970).
7. LONG, E. R.: In LONG, E. R. (ed.): *The Chemistry and Chemotherapy of Tuberculosis*, 3rd ed. Waverly Press, Baltimore 1958. pp. 119-121.
8. LURIA, S. E.: In GUNSALUS, I. C. STAINER, R. Y. (ed.): *The Bacteria*. Vol. 1. Structure. Academic Press, New York 1960.
9. MÖLLERS, B. cit. by KRAUSS, R., SIEBERT: In KOLLE, W., KRAUSS, R., UHLENHUTH, (eds.): *Handbuch der Pathogenen Mikroorganismen*. Vol. 5. 3rd ed. Urban and Schwarzenberg, Berlin 1929.
10. PORTER, J. R.: In PORTER, J. R. (ed.): *Bacterial Chemistry and Physiology*. Wiley, New York 1946. pp. 352-441.
11. CROUTHAMER, C. E., ADAMS, F., DAVIS, R.: *Applied Gamma-Ray Spectrometry*. Pergamon Press, Oxford 1970.
12. RIBBONS, D. W.: In NORRIS, J. R., RIBBONS, D. W. (eds.): *Methods in Microbiology*. Vol. 3A. Academic Press, New York 1971. pp. 297-304.
13. ROUF, M. A.: *J. Bact.* **6**, 1545 (1964).
14. SZABÓ, I., VANDRA, E.: *Acta microbiol. Acad. Sci. hung.* **10**, 213 (1963).
15. FRIEDMAN, H. C., CAGEN, L. M.: *Ann. Rev. Microbiol.* **24**, 159 (1970).
16. DREA, W. F.: *Amer. Rev. Tuberc.* **74**, 145 (1956).
17. SNOW, G. A.: *Bact. Rev.* **34**, 99 (1970).
18. WILLISTON, E. H., BINGENHEIMER, J., ROSENTHAL, S. R.: *Ann. Inst. Pasteur* **94**, 49 (1953).
19. HUTNER, S. H.: *Ann. Rev. Microbiol.* **26**, 313 (1972).
20. WEINBERG, E. D.: *Bact. Rev.* **1**, 136 (1966).

Address of the authors:

VALENTINA KARASSEVA
Microbiological Research Group, Hungarian Academy of Sciences
Pihenő út 1, H-1529 Budapest, Hungary
MARIA ÖRDÖGH, ILONA SZIKLAI
Central Research Institute for Physics, Hungarian Academy of Sciences
Konkoly Thege M. út, H-1121 Budapest, Hungary

COMPARATIVE STUDIES ON THE SOLUBLE PROTEINS OF ADENOVIRUS TYPE 1

ÉVA ÁDÁM, I. NÁSZ and P. MEDVECZKY

Institute of Microbiology, Semmelweis University Medical School, Budapest

(Received August 25, 1976)

The soluble proteins of adenovirus type 1 have been separated and purified. Their antigenic characteristics were compared in different precipitation experiments performed in electric field. Both two-dimension immune electrophoresis and rocket electrophoresis can successfully be applied for quick diagnostic purposes. Quantitative determination of virus proteins is also feasible by rocket electrophoresis. The isoelectric point values of hexon, penton and fibre were pI 4.55, pI 4.69 and pI 7.07, respectively. The amino acid composition of type 1 adenovirus and its capsid components was determined from separated and purified protein preparations. The former differed in amino acid composition from the tissues used for virus propagation.

Adenoviruses of icosahedral symmetry contain protein besides their double stranded linear DNA. The hexon, penton base and its projection, the fibre [1] the constituents of the protein capsid enveloping the nucleic acid, amount to 80% of the total protein, while the remaining 20% comprises the DNA bound core or inner protein [2].

In the course of the replication of adenoviruses in sensitive cells, different virus proteins are produced in large excess, besides the complete virion. According to EVERITT *et al.* [3] 1–5% of the penton and fibre and 20–30% of the hexon incorporates in the new virus population, and the remaining part persists in the infected cells or in their supernatant in soluble form.

Knowledge is deficient concerning the type 1 adenovirus hexon, penton and fibre antigens, their chromatographic characteristics as well as their electrophoretic mobility in different systems. Isoelectric point and amino acid composition of the three virus proteins are also unknown. Determination of these data for purified structure proteins and type 1 adenovirion is described below.

Materials and methods

Tissue cultures, virus propagation. Prototype 1 adenovirus strain was propagated in permanent human HEp-2 and AV₃ (amnion) cell lines as described earlier [4]. Infected cells were separated from the maintenance solution by centrifugation at 2000 rpm, and disintegrated in an MSE ultrasonic desintegrator.

Separation and purification of soluble components and virions. 1. *Ultracentrifugation.* Virions were separated from the soluble components in an MSE Superspeed 50 ultracentrifuge (3 × 20 ml swing-out rotor 27 000 rpm, 1.5–4 hr) on a double CsCl cushion of 1.34 and 1.45 g/ml density. The fractions corresponding to 1.34 g/ml density, which contained the complete virions, were subjected to equilibrium ultracentrifugation and concentration (3 × 20 ml rotor 27 000 rpm, 24–48 hr). The soluble virus proteins were separated from the fraction situated above the virion line.

2. *Anion exchange chromatography.* Elution of virus proteins from DEAE Sephadex A-50 column (Pharmacia) was performed as described earlier [5].

3. *Gel filtration.* Separated components were filtrated through a G-200 column (Pharmacia). A 0.01 M phosphate buffer pH 7.0 containing 0.5 M NaCl and 0.02% NaN₃ as antibacterial agent was used as eluent. The characteristic parameters of the column (V_0 , V_t) as well as separation according to molecular weight were determined by using Dextran Blue (Pharmacia), crystalline RNase (Sigma), BSA V fraction (Fluka) and phenol red.

Determination of the isoelectric point. The isoelectric point of the purified components was determined in a 110 ml LKB 8100 Ampholine Electrofocusing Equipment. Materials were dialysed against 1% glycine solution for 72 hr before the experiment. A 0–46% (w/v) sucrose solution was added for stabilizing the ampholite gradient in the 3–10 pH range. The anode buffer contained 2% phosphoric acid and the cathode buffer 1.5% NaOH. The isoelectric point was determined at 300 V during 72 hr while the intensity dropped from 0.5 A to 0.005 A. Fractions were collected with an LKB Ultrarac automatic fraction collector. The protein content was determined with an Uvicord II absorptiometer, the pH with a Radelkis OP 205 pH-meter.

Antigen-antibody reactions. Immune sera were prepared in rabbits against complete virus pool and separated components, using Freund's complete adjuvant. When an appropriate antibody level had developed, the rabbits were bled by heart puncture.

1. *Immune osmophoresis, immune electrophoresis.* The behaviour of virus antigens in the electric field was studied in 1% agarose gel (SeaKemTM) prepared in 0.15 M phosphate buffer pH 7.0. Experimental conditions were as described earlier [6].

2. *Two-dimension immune electrophoresis.* In the first step the antigens were separated in 1% agarose gel by electric current for 3–3.5 hr. Subsequently the immune serum was placed in wells cut parallel with the direction of migration and run after having turned the plates by 90°. In this position the immune serum was situated towards the positive pole. Migration time was 2.5–3 hr.

Rocket electrophoresis. Ten per cent immune serum was mixed to agarose, dissolved and then cooled to 50°C. Wells were cut in the middle of the plate and filled with 10–50 µl virus protein containing solution. Electrophoresis was carried out at 40 V for 3–6 hr.

Trypsin treatment. The virus pool or purified virus proteins were treated with twice crystallized trypsin (Merck) of 0.1% end concentration at 37°C for 1 hr. The trypsin effect was stopped by an identical quantity of trypsin inhibitor (Sigma, St. Louis).

Protein determination. Protein concentration was determined according to LOWRY et al. [7] using a PYE Unicam SP 800 spectrophotometer.

Polyacrylamide-gel electrophoresis. The technique described earlier [5] was modified by staining the gels with Amidoschwarz 10 B (Serva). Electropherograms were evaluated in a Kipp Zonen (Delft, Holland) densitometer. Virus antigen containing solutions were concentrated 2–5 times in air stream.

Amino acid composition determination. The amino acid composition of the purified and concentrated hexon, penton, fibre and virion total protein as well as that of the soluble proteins of HEp-2 and AV₃ tissues disintegrated similarly as were the infected cells, was determined. The samples were hydrolysed in 6 N HCl at 108–110°C and examined in a JLC-5 AH (Jeol) automatic amino acid analyser.

Results

Preparation of purified soluble proteins

The soluble adenovirus antigens were separated from the virion by ultracentrifugation. In anion exchange chromatography on DEAE Sephadex A-50 column, part of the fibre antigen was eluted with 0.01 M phosphate buffer and the rest with buffer of 0.025–0.1 M NaCl content. In the second peak the penton was eluted from the column between 0.3–0.4 M NaCl content and finally the hexon between 0.475–0.6 M (Fig. 1). The "width" of the elution range depended on the quantity of the protein placed on the column. Several repetitions of the chromatography did not influence significantly the concentra-

tion of NaCl required for elution. After anion exchange chromatography, gel filtration was performed on Sephadex G-200 column. The homogeneity of the preparations after chromatography and gel filtration was controlled immunologically by polyacrylamide-gel electrophoresis and electron microscopy, and only homogeneous preparations were used in subsequent experiments.

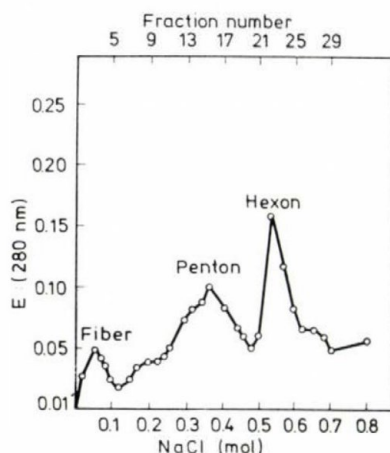


Fig. 1. Elution of the soluble proteins of type 1 adenovirus from DEAE Sephadex A-50 column

Isoelectric point of soluble adenovirus proteins

The homogeneous hexon, penton and fibre preparations were subjected to isoelectric focussing for 72 hr. During this time the proteins gathered in the form of opalescent disks in the pH range corresponding to their isoelectric point. Having collected the fractions and determined the pH of the fractions, the protein i.e. the virus antigen content was studied with gel diffusion, using specific immune sera. Considering the peak of the fractions which gave precipitation lines and the pH values, the isoelectric point of adenovirus type 1 hexon was pI 4.55; that of the penton, pI 4.69; and of the fibre, 7.07.

Behaviour of soluble components in the electric field

1. *Immune electrophoresis.* Hexon migrated towards the anode, the fibre towards the cathode, and the penton, though essentially slower than the hexon, likewise towards the positive pole. After 3–3.5 hr required for the separation of the antigens, the immune sera were applied to the wells cut parallel to the direction of the current. Thus the whole examination time was 24–48 hr. Fig. 2 shows the results of this experiment. After trypsin treatment the central precipitation-line of the penton antigen disappeared in immune electrophoresis, indicating the trypsin sensitivity of the penton base. Hexon

and fibre antigens failed to show any change upon trypsinization. Storage at -20°C for 2 years did not influence the antigenicity of the hexon neither did the 6-month storage affect that of the fibre and the penton.

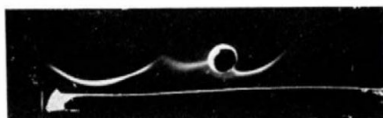


Fig. 2. Immune electrophoresis of type 1 adenovirus pool

2. *Immune osmophoresis.* In immun osmophoresis the antigen and antibody are exposed simultaneously to the effect of electric current, thus essentially less time is required for the appearance of the precipitation lines. The precipitation line of the hexon takes 30–60 min to become visible while those of the fibre and penton take 2–3 hr.

Determining the protein content of the hexon preparations by the method of LOWRY *et al.* [7], it was found that $0.1\text{ }\mu\text{g}$ hexone in $10\text{ }\mu\text{l}$ total volume gave a well visible precipitation line (Fig. 3).

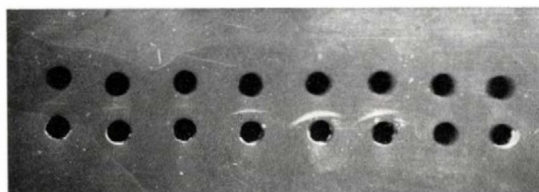


Fig. 3. Immune osmophoresis of type 1 adenovirus hexon. Upper row: 0.1 , 0.2 , 0.3 , $0.4\text{ }\mu\text{g}$ hexon, physiological saline, $0.4\text{ }\mu\text{g}$ hexon. Lower row: antihexon immune serum in wells 1–7, physiological saline in well 8

3. *Two-dimension immune electrophoresis.* Immune electrophoresis is a suitable method for the separation of virus antigens according to their electric charge, and immune osmophoresis requires little time. Thus their combined application allows a satisfactory separation in 6–8 hr. Separation of the virus antigens takes place in the first step and after placing the immune serum in the wells, the plate is again exposed to the current, so that the antibody migrates towards the negative pole. Naturally, the antigens keep moving as well. Thus, in contrast to the pattern developing in immune electrophoresis where the precipitation lines are situated parallel to the wells of immune sera, in two-dimension immune electrophoresis the precipitation line of the hexon appears nearer to, and that of the fibre farther of, the immune serum well [Fig. 4].

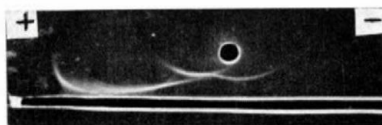


Fig. 4. Two-dimension immune electrophoresis of soluble adenovirus antigens

4. *Rocket electrophoresis.* In the immune serum containing agarose, under current effect the antigen meets immediately the immune serum and the formation of precipitation areas can be followed. The size of the precipitation area of the hexon migrating towards the anode does not exceed the diameter of the antigen reservoir and that of the penton migrating also to the positive pole appears in the form of a homogeneous opalescence. The fibre surrounds arch-like eccentrically the antigen reservoir with the concave side towards the cathode (Fig. 5).

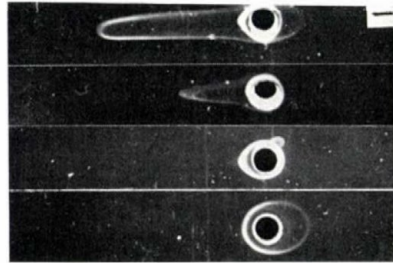


Fig. 5. Rocket electrophoresis of type 1 adenovirus pool and separated hexon, penton and fibre antigens in 10% antipool immune serum containing agarose gel

These examinations require 3–7 hr depending on the concentration of the antigen, thus they are most suitable for the quick determination of virus proteins.

While in immune electrophoresis, immune osmophoresis and two-dimension immune electrophoresis, the size and intensity of the precipitation line are only slightly influenced by the quantity of antigen, in rocket electrophoresis the precipitation area is, similarly as that of other proteins [8], directly proportional to the concentration of the virus antigen. The same was found in experiments performed with 1 : 2 dilutions of hexon preparations. Ten μl of hexon preparation of 250 $\mu\text{g/ml}$, 500 $\mu\text{g/ml}$, 1000 $\mu\text{g/ml}$ and 2000 $\mu\text{g/ml}$ concentration were placed in the antigen reservoir for 5 hr. Figure 6 shows the developed precipitation pattern. With rocket electrophoresis of hexon solutions of known and unknown concentration, the unknown virus protein concentration could be determined.

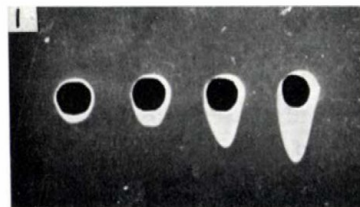


Fig. 6. Rocket electrophoresis of purified hexon preparations. Amount of antigen, 10 μl ; protein concentration, 250 $\mu\text{g/ml}$; 500 $\mu\text{g/ml}$, 1000 $\mu\text{g/ml}$ and 2000 $\mu\text{g/ml}$

Amino acid concentration of capsid proteins

Results are summarized in Table I. Studying the amino acid composition of the soluble components of type 1 adenovirus, the aspartic and glutamic acid concentrations were higher in the acid hydrolysate of hexon than of penton and fibre, whereas the penton and fibre antigens contained a higher per cent of basic amino acid (lysine) than the hexon. Methionine was present in traces in all samples, cystine could not be detected. Acid hydrolysis was not suitable for the determination of tryptophan.

Table I

Amino acid composition of structure proteins and virion of adenovirus type 1

Amino acid*	Hexon	Penton	Fibre	Virion	HEp-2	AV ₃
Lys	4.07	5.04	6.13	6.34	14.86	17.61
His	2.05	2.03	1.36	2.11	traces	traces
Arg	4.72	3.80	2.01	7.99	2.35	1.50
Asp	15.30	13.19	13.10	11.14	10.68	8.39
Thr	6.62	8.23	10.40	6.90	5.58	4.67
Ser	7.85	9.47	12.10	6.86	6.95	5.60
Glu	10.30	10.14	7.13	9.86	14.70	20.00
Pro	8.87	7.01	6.69	7.30	5.62	5.30
Gly	7.56	7.69	8.92	7.45	7.05	6.50
Ala	7.50	7.69	7.18	8.40	8.30	7.10
Val	5.10	5.72	6.00	5.69	5.20	4.30
Met	traces	traces	traces	traces	traces	2.50
Ile	2.89	2.91	2.58	3.17	3.00	2.98
Leu	6.66	8.28	8.61	7.30	8.10	6.81
Tyr	4.30	2.67	2.24	3.12	2.71	2.25
Phe	4.09	3.78	2.51	3.68	3.52	2.71
Trp	ND	ND	ND	ND	ND	ND
1/2 Cys	ND	ND	ND	ND	traces	traces

* Amino acid compositions expressed in mol/100 mol amino acid

ND = Not detected or not determined

Discussion

The separated and purified soluble antigens of type 1 adenovirus have been studied in the electric field with different methods. Comparing the results obtained with immune osmophoresis, immune electrophoresis, two-dimension immune electrophoresis and rocket electrophoresis, two-dimension immune electrophoresis and rocket electrophoresis proved the best for the identification

of virus antigens and for quick diagnostic purposes, owing to the satisfactory separation and the short examination time. According to data in the literature in rocket electrophoresis the size of the precipitation line is directly proportional to the quantity of the protein [8]. Rocket electrophoresis of hexon preparations of known and unknown virus antigen content appears to be suitable for the quantitative determination of virus protein. Amino acid analysis of type 1 adenovirion, separated and purified hexon, penton and fibre preparations, as well as that of the acid hydrolysate of HEP-2 and AV₃ cells used for virus propagation revealed differences in the amino acid composition of the tissues, while the virion and soluble components displayed no significant deviation in this respect except for the higher arginine content of the virion. This observation is explained in other adenovirus types by the presence of an arginine-rich inner core protein [9]. Differences were found in the aspartic and glutamic acid contents, too. The aspartic and glutamic acid contents were higher in hexon and lower in penton and fibre. The ratio of basic amino acids was higher in penton and the fibre. These results were in good agreement with the data for the type 2 virion and its soluble components [10-13].

Few data are available concerning the isoelectric point of the soluble components of adenoviruses and none concerning type 1 [14]. In the present work the isoelectric point of the three soluble antigens of adenovirus type 1 has been determined. That of hexon and penton was found in the acid pH range and of the fibre at a slightly alkaline pH value.

Acknowledgements. We are indebted to Miss VERONIKA GARAMVÖLGYI for excellent technical assistance, and to the staff of the First Institute of Chemistry and Biochemistry, Semmelweis University Medical School, Budapest, for kind help in amino acid analysis.

REFERENCES

1. GINSBERG, H. S., PEREIRA, H. G., VALENTINE, R. C., WILCOX, W. C.: *Virology* **28**, 782 (1966).
2. LAVER, W. G.: *Virology* **41**, 488 (1970).
3. EVERITT, E., SUNDQUIST, B., PHILIPSON, L.: *Virology* **8**, 742 (1971).
4. ÁDÁM, É., LENGYEL, A., NÁSZ, I.: *Kísérlet. Orvostud.* **27**, 612 (1975).
5. NÁSZ, I., LENGYEL, A., CSERBA, I.: *Orvostudomány* **22**, 101 (1971).
6. NÁSZ, I., CSERBA, I., RÓZSA, K.: *Orvostudomány* **19**, 55 (1967).
7. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., RANDALL, R. J.: *J. biol. Chem.* **193**, 265 (1951).
8. RENN, D. W., EVANS, E.: *Anal. Biochem.* **71**, 588 (1976).
9. POLASA, H., GREEN, M.: *Virology* **31**, 565 (1967).
10. BOULANGER, P. A., FLAMENCOURT, P., BISERTE, G.: *Eur. J. Biochem.* **10**, 116 (1969).
11. PETTERSON, U., PHILIPSON, L., HÖGLUND, S.: *Virology* **33**, 575 (1967).
12. PETTERSON, U., PHILIPSON, S., HÖGLUND, S.: *Virology* **35**, 204 (1968).
13. PETTERSON, U., HÖGLUND, S.: *Virology* **39**, 90 (1969).
14. PHILIPSON, L., PETTERSON, U., LINDBERG, U.: *Molecular Biology of Adenoviruses*. Springer Verlag, Berlin 1975.

Address of the authors:

ÉVA ÁDÁM, ISTVÁN NÁSZ, PÉTER MEDVECKY
Institute of Microbiology, Semmelweis University Medical School
Högyes Endre u. 7-9, H-1092 Budapest, Hungary

THE USE OF ION EXCHANGE CHROMATOGRAPHY FOR DEMONSTRATION OF RUBELLA-SPECIFIC IgM ANTIBODIES

G. NAGY and ILONA MEZEY

National Institute of Hygiene, Budapest

(Received August 30, 1976)

IgM and IgG immunoglobulins of human sera were separated by stepwise column chromatography in QAE-Sephadex A-25 ion exchanger gel bed. The procedure resulted within 30 min in a fraction suitable for direct titration of rubella-specific IgM antibodies by haemagglutination inhibition test. The method proved to be a useful diagnostic tool for primary rubella. Serum samples of 13 individuals with previously acquired immunity, 152 patients with a recent rubella-like illness, and 194 pregnant women exposed to rubella infection were tested for the presence of rubella-specific IgM antibodies. Sera of individuals with previous immunity proved to be negative for specific IgM antibodies. Specific IgM titre was demonstrated in the blood of all the 25 patients with significant titre-rise tested because of rubella-like illness, and also in the sera of additional 8 patients whose serum samples were taken too late for demonstration of a rise in titre. Significant titre-rises were found in 5 women exposed to rubella infection, but only two of them exhibited rubella-specific IgM antibodies. The absence of specific IgM antibodies refers presumably to subclinical reinfection in the other three cases.

Well-timed antibody tests may help to confirm or rule out recent rubella infection in patients with rubella-like disease or with a history of exposure to rubella. Actual infection can be detected by demonstration of a significant titre-rise of rubella haemagglutination inhibiting (HI) antibodies in paired sera [1]. The method, however, fails when the first blood sample is not taken within 3–5 days after onset of illness or within 7–14 days after contact with a patient with rubella. HI antibodies detected in serum samples taken after the above-mentioned period may indicate either a past or a most recent infection. It is also known that there is a limited chance for interpretation of serological findings when the exact time of exposure to rubella is not available.

It is generally accepted that rubella-specific IgM antibodies are present in the blood mostly for one or two months after primary infection [2–6]. Their detection may indicate a recent infection even in cases when HI or other tests are no longer informative.

Difficulties in the interpretation of serological findings are frequent in the course of rubella diagnostics. For this reason, introduction of a suitable method for demonstration of rubella-specific IgM antibodies seemed to be important. Considering the practical value of methods applied for such pur-

poses and the available technical possibilities, we have adopted the separation of IgM immunoglobulins of sera by ion exchange chromatography, as a new aid to the diagnosis of primary rubella.

The different immunoglobulin classes are well-known to elute in separate but partially overlapping fractions on ion exchanger gel beds when a continuous gradient is used [7]. As the binding of IgM to anion exchanger gels is the most intensive of the three main immunoglobulin classes, we have attempted to isolate IgM antibodies in the second step of a two-step gradient after eluting IgG and IgA antibodies with the starting buffer. The present paper gives an account of the development and the results of the method.

Materials and methods

Sera examined. Serum samples were collected from 13 individuals who had acquired immunity more than one year before, based on repeated serological tests.

From our routine diagnostic materials, 365 rubella HI positive serum samples of 346 subjects were selected for chromatography. Of the subjects 302 (87%) were pregnant, being mostly in the first trimester of gestation. Of the patients 152 were tested because of a recent rubella-like illness, and 194 subjects because of contact with a suspected case of rubella.

Sera examined within a week following receipt were kept at +4°C, the others were stored at -20°C until examination. All the samples were tested without heat inactivation.

Elimination of non-specific serum inhibitors and agglutinins. For preliminary treatment, 0.4 ml serum was subjected to heparin-manganous chloride treatment and absorption with pigeon erythrocytes prior to chromatography. The procedure was performed essentially according to the description published by the Center for Disease Control, Atlanta, Georgia, U.S.A. [8-10]. A slight modification was the omission of the last dilution step in order to reduce final dilution of the serum. The pretreated serum was desalted by gel filtration on a 1.2 × 13 cm Sephadex G-25 bed (Pharmacia Fine Chemicals AB, Sweden) using 0.1 M tris-HCl buffer, pH 8.1, as eluent in order to obtain the same ionic composition of the sample as that of the starting buffer of the ion exchange chromatography.

Ion exchange chromatography. Material of high molecular weight eluting in the void volume on Sephadex G-25 gel was applied to a 0.8 × 25 cm bed of QAE-Sephadex A-25 equilibrated by 0.1 M tris-HCl buffer, pH 8.1. Ion exchange chromatography was performed stepwise with the same buffer as the starting one for elution of the first peak. The starting buffer eluted some IgA and almost the whole mass, about 98-99%, of IgG immunoglobulins from the gel. The second peak, containing almost exclusively IgM immunoglobulins, was obtained by replacing the first buffer by 0.3 M tris-HCl buffer, pH 6.6. Elution curves were detected at 280 nm wave-length by running the eluate through an LKB Uvicord II ultraviolet absorptiometer (LKB Producter AB, Sweden). Fractions of about 1.0 ml volume were collected from the peaks.

Haemagglutination inhibition (HI) test. Investigation of rubella HI antibody titres of the fractions obtained in ion exchange chromatography were conducted by the CDC Standard Rubella Haemagglutination Inhibition Test [10].

Ion exchange chromatography of ABO blood group type sera. Human ABO blood group typing "Serotyp" sera (Institute for Serobacteriological Production and Research, Human, Budapest) were submitted to ion exchange chromatography in order to determine the distribution and dilution of the IgM antibodies. Isohaemagglutinin titration of the "Serotyp" fractions was performed on TAKÁTSY's microtitrator plates against the corresponding type of human red blood cells. The procedure was applied throughout as a reliable control of the constant conditions. According to these experiments the IgM antibodies of 0.4 ml serum showed about four to eight-fold dilution in the top fraction of the second protein peak.

Immunodiffusion. The distribution of immunoglobulin classes in the fractions obtained by ion exchange chromatography were frequently tested by immunodiffusion in the course of the experiments. Anti-human IgG (heavy chain specific, Lot 65 773), anti-human IgA (Lot 46 242) and anti-human IgM (heavy chain specific, Lot 63 324) goat sera (Cappel Laboratories Inc., Downington, Pennsylvania, U.S.A.) were used in these tests. Immunodiffusion was

performed applying 0.9% agar gel (Difco Laboratories, Detroit, Michigan, U.S.A.) on 5×5 cm glass slides. Tests were incubated for 48–72 hr at room temperature and stained with Coomassie Brilliant Blue R-250 (Serva Feinbiochemica, Heidelberg, G.F.R.) if a permanent record was desired. Immunodiffusion plates have shown in the IgM fraction besides some IgA immunoglobulins, only a slight trailing of IgG immunoglobulins. This high grade separation allowed the detection of an IgM antibody titre as low as 1 : 16 of the original serum.

2-Mercaptoethanol (2-ME) treatment. The IgM fraction of ion exchange chromatography showing a significant HI titre was submitted to 2-ME treatment. Of the fraction, 0.45 ml was mixed with 0.05 ml of 0.5 M 2-ME freshly prepared in phosphate-buffered saline, pH 7.5, on the day of the experiment. The same volume of the fraction was supplemented with 0.05 ml of saline. Samples were tested for HI antibodies after incubation at 37°C for one hour. Only those titres were considered to represent rubella specific IgM antibodies which showed an at least fourfold decrease after 2-ME treatment.

Results

Examination of sera from individuals with existing immunity. When sera of 13 individuals with a history of previously acquired immunity were submitted to ion exchange chromatography, no rubella HI titres were found in the IgM fraction, except for one. In this exceptional case a low HI titre could be demonstrated. This fraction could, however, be considered negative for rubella-specific IgM antibodies, since the detected activity remained unchanged after 2-ME treatment.

Examination of sera from patients with rubella-like illness (Table I). The clinical diagnosis of rubella was confirmed by a significant rise in rubella-specific HI antibody titre in the case of 25 members of the group. The presence of rubella-specific IgM antibodies was demonstrated in each of their 33 rubella antibody positive serum samples.

There were 127 patients whose first serum samples were taken too late for demonstration of a rise in titre after the onset of illness and contained high titre rubella HI antibodies. In 11 cases the time-delay was more than four weeks after appearance of the rash. Rubella-specific IgM antibodies were demonstrated in serum samples of eight of the 127 patients, whereas a 2-ME resistant HI titre could be found in the IgM fraction of one patient. In 8 patients, the lack of specific IgM antibodies failed to rule out actual rubella infection because of the time-delay over four weeks.

Table I

Results of tests with sera taken from patients with rubella-like illness

	Significant rise in rubella HI titre		Total
	detected	not detected	
IgM positive	25	8	33
IgM negative	—	119	119
Total	25	127	152

Examination of sera taken from pregnant women exposed to rubella infection (Table II). Members of this group did not show clinical signs of rubella. A significant titre-rise was found in the case of five women. Rubella-specific IgM antibodies were, however, demonstrated in two cases only. In spite of the considerable titre-rise observed (1 : 32–1 : 1024) chromatography of both the early and the late sera of three individuals indicated only the presence of rubella-specific IgG antibodies.

Table II
Results of tests with sera taken from subjects exposed to rubella

	Significant rise in rubella HI titre		Total
	detected	not detected	
IgM positive	2	—	2
IgM negative	3	189	192
Total	5	189	194

Sera of 51 members of this group showed a high level of rubella HI antibodies (over 1 : 256) some days after contact with a suspected case of rubella. The absence of rubella-specific IgM antibodies in their blood indicated that their immunity originated from an infection contracted more than one month before examination.

Rubella HI antibody positive first serum samples of 138 subjects exposed to rubella were taken too late for demonstration of a significant titre-rise. All these sera were negative for rubella-specific IgM antibodies. Actual sub-clinical infection could not be ruled out in the case of 21 individuals whose serum samples had been taken over 6 weeks following contact.

Discussion

The presence of rubella-specific IgM antibodies in sera may be detected by several methods, *viz.* (a) Decomposition of IgM immunoglobulins by treatment of whole serum with 2-ME and demonstration of a significant decrease in rubella HI antibody titre [2]. (b) Indirect immunofluorescence technique using anti-human IgM conjugate [5, 11–14]. (c) Separation of IgM and IgG immunoglobulins of serum and demonstration of rubella HI activity in the IgM fraction. Procedures described for serum fractionation include (i) sucrose density gradient centrifugation [3, 4, 13–15] and (ii) permeation chromatography on Sephadex G-200, [6, 16–18] on agarose [19] and on controlled pore glass [20] columns.

The 2-ME treatment of whole serum is an unsatisfactory method, since its usefulness is limited to early acute-phase of illness when the level of specific

IgG antibodies does not surpass that of IgM antibodies [13]. Though the other methods mentioned above offer a much wider range of application, each of them has some disadvantages.

Ion exchange chromatography has been used previously only as an additional step to other techniques of rubella serology [21–23]. Our present investigations have, however, shown that ion exchange column chromatography of the serum gives at least the same quality of separation of IgM immunoglobulins from IgG ones as density gradient centrifugation or gel filtration and at the same time it is significantly less time-consuming than the mentioned other methods. Fractions obtained by ion exchange chromatography are suitable for direct titration of specific IgM antibodies by the generally used HI test. This is an advantage for laboratories which are not equipped for immunofluorescence tests. It is, however, a disadvantage that the use of an absorptiometer is needed for the detection of the protein peaks. Preliminary investigations show that this problem can be overcome by using a batch technique instead of column chromatography.

Recently it has been reported by several authors that after heparin-manganous chloride treatment lipoproteins with HI activity may remain in the sera [24, 25]. Failure of heparin-manganous chloride treatment may result in false positive rubella HI titres in the IgM fraction. This difficulty can be overcome by control of sensitivity of HI substance to 2-ME treatment. False HI titres of the IgM fraction demonstrated in the course of the present investigations were probably due to non-specific inhibitors.

An increasing number of papers deals with the problem of rubella reinfection suggesting that in the course of a rubella epidemic those members of the population who possess a low antibody level may contract a repeated and mostly subclinical infection [26–33]. In the course of the present study, a diagnostic rise in rubella HI titre without demonstrable specific IgM antibodies was found in three individuals without clinical signs of rubella. The observed antibody response was probably due to subclinical reinfection.

Acknowledgements. We are indebted to Dr. M. SIMON for advices and help in testing the quality of separation, and to Drs E. MOLNÁR and I. DÖMÖK for helpful criticism during preparation of the manuscript. Thanks are due to Mrs. E. GÁSPÁR and Mrs. Zs. PALÁNSZKY for excellent technical assistance.

REFERENCES

1. STEWART, G. L., PARKMAN, P. D., HOPPS, H. E., DOUGLAS, R. D., HAMILTON, J. P., MEYER, H. M. JR.: *New Engl. J. Med.* **276** 554 (1967).
2. BANATVALA, J. E., BEST, J. M., KENNEDY, E. A., SMITH, E. E., SPENCE, M. E.: *Brit. med. J.* **3**, 285 (1967).
3. VESIKARI, T., VAHERI, A.: *Brit. med. J.* **1**, 221 (1968).
4. OGRA, P. L., KERR-GRANT, D., UMANA, G., DZIERBA, J., WEINTRAUB, D.: *New Engl. J. Med.* **285**, 1333 (1971).
5. HAIRE, M., HADDEN, D. S. M.: *J. med. Microbiol.* **5**, 237 (1972).

6. PATTISON, J. R., DANE, D. S., MACE, J. E.: *Lancet* **1**, 185 (1974).
7. KAWAMURA, A. JR. (ed.): *Fluorescent Antibody Techniques and their Application*. University of Tokyo Press, Tokyo 1969. p. 46.
8. PEETERMANS, J., HUYGELEN, G.: *Presse méd.* **75**, 2177 (1967).
9. FELDMAN, H. A.: *Proc. Soc. exp. Biol. (N. Y.)* **127**, 570 (1968).
10. A standardized rubella HAI test. Immunology Series No. 2. Procedural Guide. Center for Disease Control. Atlanta, Georgia 1970.
11. BAUBLIS, J. V., BROWN, G. C.: *Proc. Soc. exp. Biol. (N. Y.)* **128**, 206 (1968).
12. CRADOCK-WATSON, J. E., BOURSE, M. S., VANDERVELDE, E. M.: *J. Hyg. (Lond.)* **70**, 473 (1972).
13. FORGHANI, B., SCHMIDT, N. J., LENNETTE, E. H.: *Intervirology* **1**, 48 (1973).
14. CHAGNON, A., GILKER, J. C., ROY, L., DESSUREAULT, P., PAYMENT, P.: *Intervirology* **3**, 359 (1974).
15. AL-NAKIB, W., BEST, J. M., BANATVALA, J. E.: *Lancet* **1**, 182 (1975).
16. GUPTA, J. D., PETERSON, V., STOUT, M., MURPHY, A. M.: *J. clin. Path.* **24**, 547 (1971).
17. FREYMUTH, F.: *Ann. Biol. clin. (Paris)* **30**, 579 (1972).
18. PATTISON, J. R., MACE, J. E.: *J. clin. Path.* **26**, 309 (1973).
19. BÜRGIN-WOLFF, A., HERNANDEZ, R., JUST, M.: *Lancet* **2**, 1278 (1971).
20. FRISCH-NIGGEMEYER, W.: *J. clin. Microbiol.* **2**, 377 (1975).
21. ALTEMEIER, W. A. III., MUNDON, F. K., TOP, F. H. JR., RUSSEL, P. K.: *Appl. Microbiol.* **19**, 785 (1970).
22. HORNSLETH, A., LEERHØY, J., GRAUBALLE, P., SPANGGAARD, H.: *Acta path. microbiol. scand. Sect. B.* **82**, 742 (1974).
23. HORNSGETH, A., LEERHØY, J., GRAUBALLE, P., SPANGGAARD, H.: *Infect. Immun.* **11**, 804 (1975).
24. BLUM, H., HAUKENES, G.: *Med. microbiol. Immun.* **159**, 271 (1974).
25. HAUKENES, G., BLUM, H.: *Med. microbiol. Immun.* **161**, 99 (1975).
26. HORSTMANN, D. M., LIEBHABER, H., LE BOUVIER, G. L., ROSENBERG, D. A., HALSTEAD, S. D.: *New Engl. J. Med.* **283**, 771 (1970).
27. DAVIS, W. J., LARSON, H. E., SIMSARIAN, J. P., PARKMAN, P. D., MEYER, H. M. JR.: *J. Amer. med. Ass.* **215**, 600 (1971).
28. VESIKARI, T.: *Scand. J. infect. Dis.* **4**, 11 (1972).
29. HAUKENES, G.: *Lancet* **1**, 1313 (1973).
30. HORSTMANN, D. M.: In FRIEDMAN, H., PRIER, J. E. (eds.): *Rubella*. Thomas, Springfield 1973. p. 84.
31. BANATVALA, J. E.: *Lancet* **2**, 1452 (1973).
32. EILARD, T., STRANNENGARD, Ö.: *J. infect. Dis.* **129**, 594 (1974).
33. MODLIN, J. F., BRANDLING-BENNETT, D. A., WITTE, J. J., CAMPBELL, C. C., MEYERS, J. D.: *Pediatrics* **55**, 20 (1975).

Address of the authors:

GÁBOR NAGY, ILONA MEZEY
National Institute of Hygiene
H-1966 Budapest, P.O.B. 64, Hungary

PERTUSSIS VACCINE—FACT AND FICTION*

J. CAMERON and D. W. STAINER

Connaught Laboratories Limited, Willowdale, Toronto, Canada

(Received September 16, 1976)

The present paper indicates a number of unresolved problems in the metabolism of *Bordetella pertussis* and in the standardization of pertussis vaccines. The use of chemically defined media should enable the metabolic problems to be clarified. The problems concerned with the control and standardization of vaccines can largely be resolved in the laboratory with the introduction of reference preparations but the question of the reactivity of vaccine remains for the future.

From its isolation in 1906 by BORDET and GENGOU [1] *Bordetella pertussis* has gone from being a microorganism isolated with difficulty on complex media to one which, today, can be grown in a chemically defined medium consisting simply of glutamic acid and proline, vitamins and salts [2–5]. Moreover, unlike some other microorganisms, where growth on a chemically-defined, as opposed to a richer, medium results in what might be considered an inferior microorganism, by virtue of failure to produce a particular metabolite, for example, toxin, *B. pertussis* grown in a defined medium seems to produce its full range of metabolites, certainly those of current interest such as agglutinogens, mouse-toxicity factor(s), mouse-protective antigen, histamine-sensitizing factor and lymphocytosis-promoting factor. There remain, however, a number of unresolved problems on the metabolism of *B. pertussis* which the use of defined media should help to clarify.

The explanation for the ability of *B. pertussis* to make the transition from complex to simple media seems to lie in the fact that most of the early, richer media for its isolation and growth represented mixtures of checks and balances, the checks all to a greater or lesser extent compensated for by the presence of whole blood. Peptones, for example, present in most of these media, are well-known inhibitors of *B. pertussis*, possibly by virtue of their content of colloidal sulphur and sulphides derived from sulphur-containing amino acids [6]. This particular aspect of the metabolism of *B. pertussis* was discussed by ROWATT [7].

A problem mentioned in the literature, but not pursued to any great extent, is the failure of some liquid media to support the growth of *B. pertussis*

* Presented at the Seventh Congress of the Hungarian Society of Microbiology, Budapest 1976.

when solidified [8]. MAZLOUM and ROWLEY [9] commented on this and postulated the existence of at least two different inhibitors in agar, one neutralized by charcoal, the other by catalase. In an investigation into the problem [10] it was found to break down into two parts.

1. If the liquid media contain starch, charcoal, autoclaved red blood cells or albumin, they usually support growth when solidified but not if these factors are absent.

2. If the liquid media do not contain any of these and are solidified in the presence of starch, usually 0.02 to 0.1%, the inoculum needed to initiate growth can range from 1 to 10^6 cells, depending on the agar used to solidify the media. Difco agar allows growth from single cells, Oxoid agar needs an inoculum of 10^6 cells (Fig. 1); in vaccine manufacture, where the inoculum is much in excess of 10^6 cells, the final yield of cells is usually the same regardless of the agar used and vaccines made from the cells are both equipotent and generally superior to vaccines made from cells grown in liquid culture.

The inhibitor in Oxoid agar preventing growth from small inocula was tentatively identified as manganese [10] (Fig. 2). MAZLOUM and ROWLEY [9] touched on this problem when they were unable to confirm the results of ENSMINGER *et al.* [11] who had found COHEN and WHEELER [12] medium, solidified with agar, capable of supporting growth from single-cell inocula. In retrospect, the contrary findings are possibly explained by the use of Oxoid agar by MAZLOUM and ROWLEY [9] and Difco agar by the latter workers [12].

Thus, there is still the tantalizing question of how starch and other agents interact with agar to allow growth, which would otherwise not occur, to take place when media are solidified. The problem has little direct bearing on the production of pertussis vaccine but is good evidence that agar is not the wholly inert matrix it is sometimes thought to be. Its solution, however, should lead to a better understanding of the basic metabolism of *B. pertussis*.

In 1947 POLLOCK [13] published his paper describing the growth of *B. pertussis* on media not containing blood. In the course of these experiments it was believed that *B. pertussis* had been shown to be inhibited by higher, unsaturated, fatty acids. The strains used, however, were not typical, having been adapted to grow on 10% blood agar. Conclusions drawn with such strains should obviously be verified with typical strains and the use of a chemically defined medium should make the resolution of this question quite straightforward.

Another problem not yet satisfactorily resolved is that of the size of the inoculum required to initiate growth in liquid media. This seems to be different from the question of growth on solid media: solid media, Bordet-Gengou medium, for example, can readily be formulated to support growth from a

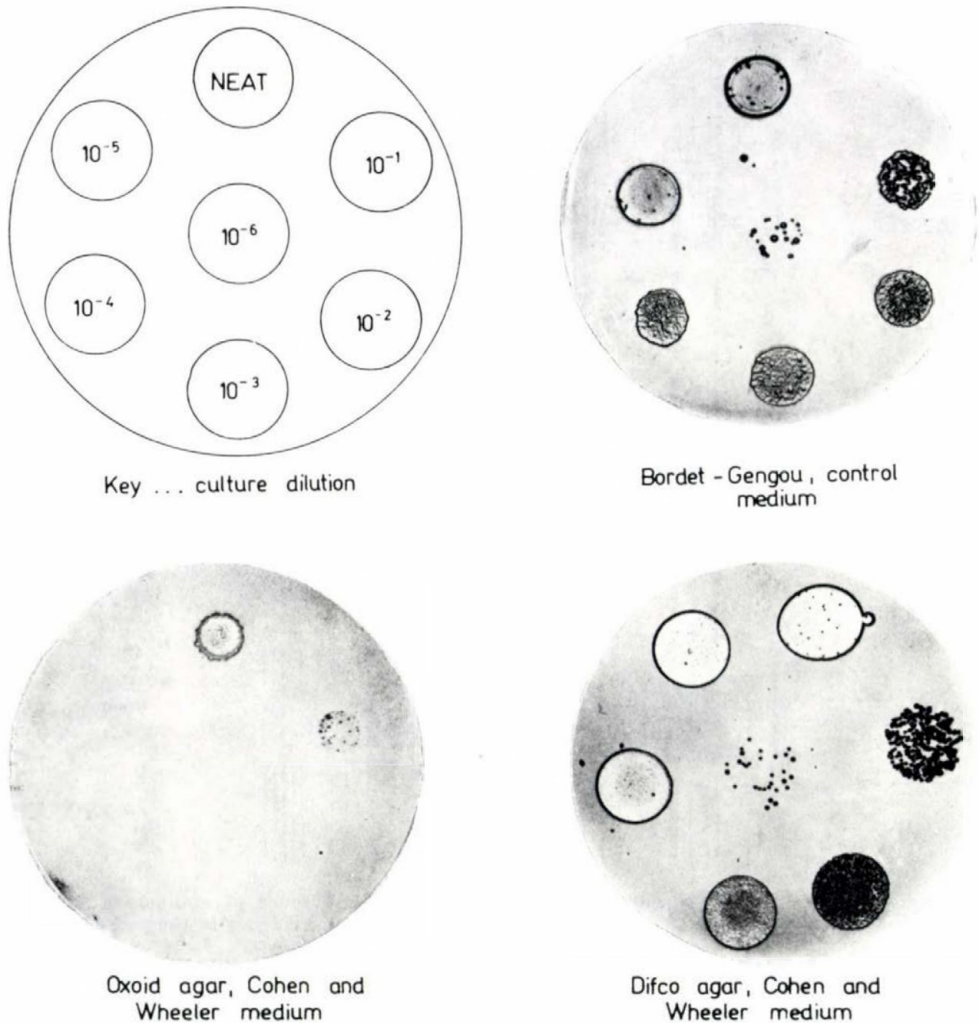


Fig. 1. Effect of agar on the growth of *B. pertussis* from different inocula; 10^{-6} dilution of culture contains 30 to 40 cells

single cell. For growth in liquid media, however, it is usually accepted that the inoculum, in cell numbers, should be between 2 and 5% of the anticipated final yield of cells. Although, as already mentioned, growth from a single cell can be obtained on Bordet-Gengou medium, and on most solid media containing an appropriate supplement, the only report, to our knowledge, of growth from a single cell in liquid culture is that of JEBB and TOMLINSON [14]. They used charcoal-purified components in their experiments but even so growth was

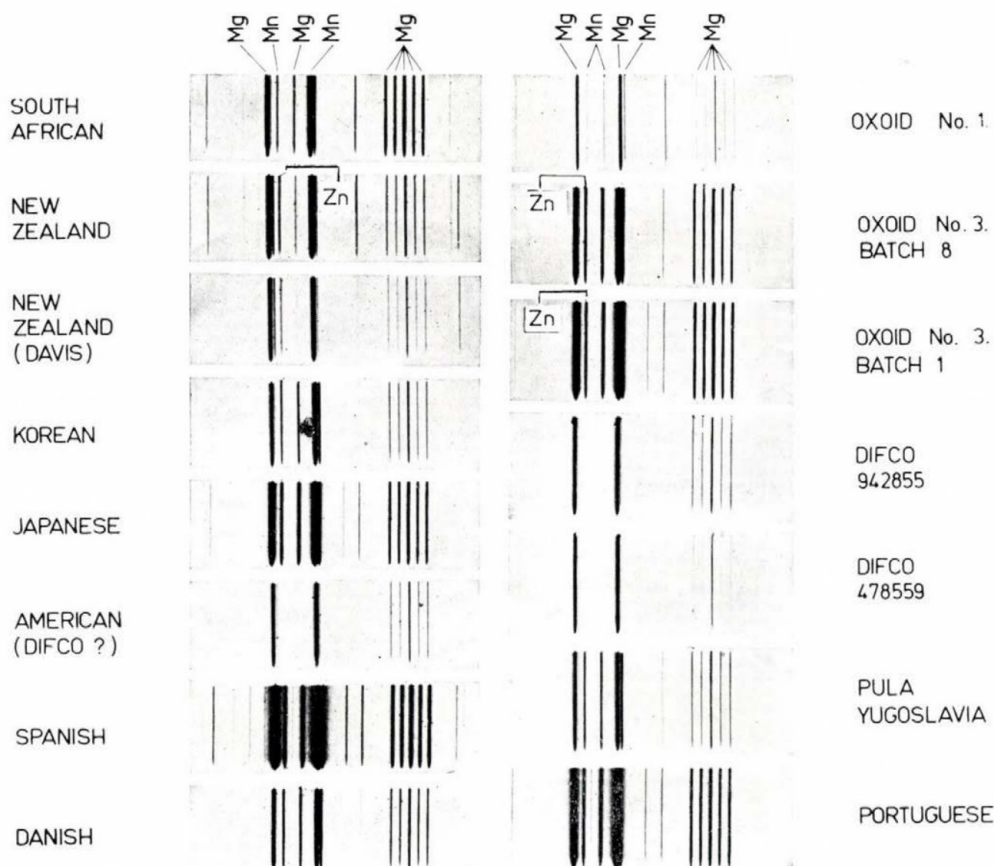


Fig. 2. Cathode-layer arc spectrograms of different agars. Absence of manganese lines in Difco and "American" (Difco?) agar. Magnesium is also less in evidence in these agars but it was found to be non-inhibitory for *B. pertussis*

both poor and slow. STAINER and SCHOLTE (personal communication) have also noted growth from single colonies in their defined medium: the lag phase was 60 hours and full growth was attained in 5 days.

Once the microorganism has been grown there is still the question as to what is a pure culture. It is a straightforward matter to look at a Bordet-Gengou plate and say, "This culture is pure", but how pure? Examination of cultures under the plate microscope reveals numerous different colonial forms (Figs 3 to 6).

What do these different forms represent? In an investigation into this question, which has now been going on for several years [15], it has been confirmed that, in addition to different colonial forms, different serotypes may be found within laboratory strains and within fresh clinical isolates [15, 16];

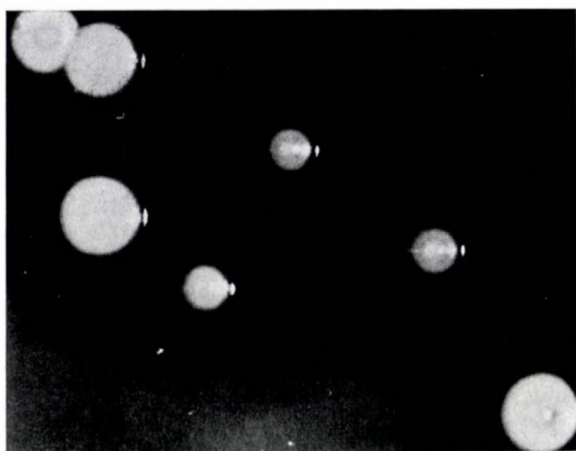


Fig. 3. Single colonies of *B. pertussis*. Note different sizes and types of colonies. Actual size of colonies, 1-2 mm

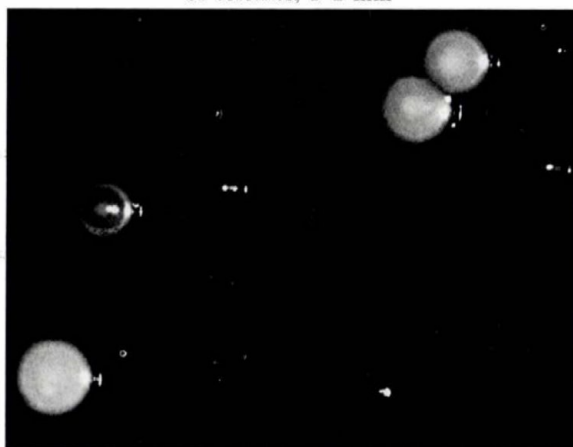


Fig. 4. Single colonies of *B. pertussis*. Different sizes and types of colonies. Actual size of colonies, 1-2 mm

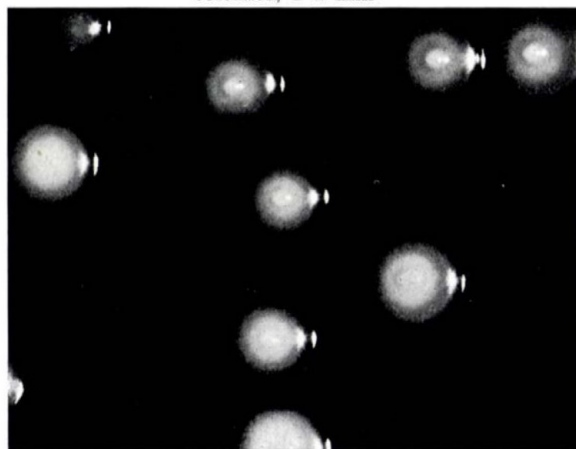


Fig. 5. Single colonies of *B. pertussis*. Note mixture of papillate and non-papillate colonies. Actual size of colonies, 1-2 mm

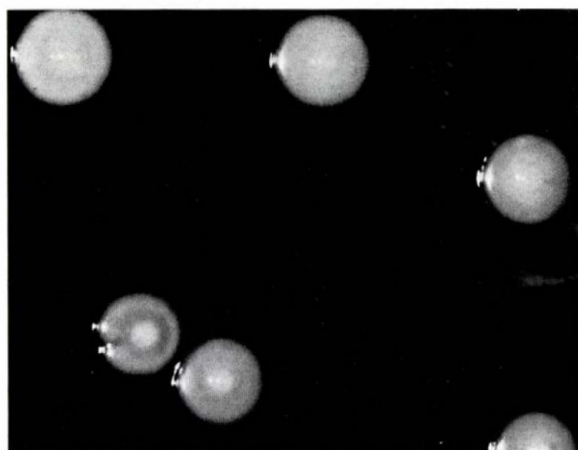


Fig. 6. Single colonies of *B. pertussis*. Note different types of marking. Actual size of colonies about 1.5 mm

also, that cloning of different colonial forms results in clones producing such different amounts of mouse-protective antigen and histamine-sensitizing factor as to suggest that, contrary to some views, these entities are not identical or, at best, are independent variables on the same molecule [15]; and that some clones, in our experience type 1 clones of vaccine strains but not of the 18 323 challenge strain, are less toxic for mice in the mouse-weight-gain test and produce less histamine-sensitizing factor than clones manifesting antigens 2 and 3 in addition to 1 [17] (Table I).

Table I

Toxicity, protective capacity and histamine sensitizing activity of colonial variants of B. pertussis

Strain	Serotype of variant	Mouse-toxic dose (number of organisms $\times 10^6$)	Protective capacity (I.U. per 20×10^9 organisms)	H.S.D. 50 (Number of organisms $\times 10^6$)	Ratio of H.S.D. 50 to protective-capacity ($\times 10^6$)
2991	1,2	10	10	0.4	0.04
	1,2	10	11	12	1.1
	1	35	4	100	25.0
2992	1,2,3	10	4	5	1.25
	1,2 (weak), 3	10	52	3	0.06
	1,2 (weak), 3	10	59	8	0.14
	1,2,3	10	23	6	0.23
4132	1,2	10	16	1	0.06
	1	40	2	100	50.0
	1,2	10	7	2	0.29
	1	40	2	20	10.0

In testing vaccines for safety and potency there are unresolved problems in the potency assay itself, in the role of agglutinins, in the effect of adjuvant and in the interpretation of the mouse-weight-gain test.

The results of EVANS and PERKINS [18, 19] with the mouse-protection test seem to be of considerable significance because of their suggestion that mice given smaller doses of vaccine will, in time, be as well protected as mice receiving larger doses (Fig. 7).

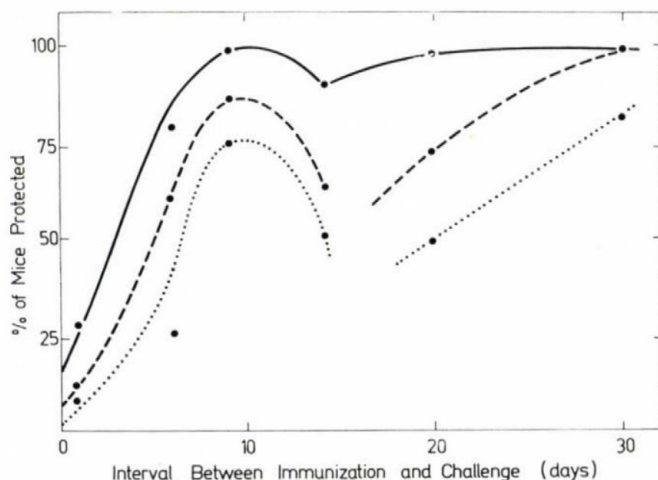


Fig. 7. Production of two immune response effects in mice following a single injection of pertussis vaccine. Vaccine 1/4 ———; vaccine 1/10 — — —; vaccine 1/25 Note suggestion that from day 20 to day 30 response to lower doses continues to rise

Do mice receiving smaller doses of vaccine indeed go on to respond as well as those receiving larger doses, as these data would imply? PITTMAN (personal communication) found this not to be so but perhaps it would be useful to repeat the experiments yet again.

There is also in mouse-protection experiments the interesting feature of the early rapid, and subsequently waning, protective response [18–20]. A similar phenomenon can be observed when lipopolysaccharide is injected into mice and the slime layer from *pseudomonas* — this factor, it should be noted, is not lipopolysaccharide. In the case of *B. pertussis*, however, the entity producing this early response is heat-labile unlike the other two. Is the protective antigen, itself heat-labile, inducing a biphasic response or are two different heat-labile entities involved?

The potency assay in mice has always presented problems for a number of reasons — poor performance by mice, difficulty in obtaining adequate numbers of mice or sheer expense, and alternatives have been sought, so far,

unsuccessfully. At one time agglutinin production in mice was considered seriously as an alternative [21, 22] but appeared to be vitiated by the performance of Pillemer's non-agglutininogenic antigen [23] in the M. R. C. trials in the United Kingdom [24-26], and was indeed rejected in favour of the mouse-protection test in the report of these trials [26]. PRESTON, however, has consistently regarded agglutinin-production as a better measure of the protective capacity of pertussis vaccines for children [27]. His grounds are twofold: that a more careful examination of responses in mice to Pillemer's antigen will indeed reveal agglutinin responses and that adjuvant enhances agglutinin responses in children [28, 29] and since adjuvant also generally enhances immune responses, agglutinin responses can be equated to protection. If applied to agglutinin responses following the injection of salmonellae the latter part of this argument is, of course, clearly invalid. Nevertheless, in the case of *B. pertussis* the argument tends to cast doubt on the value of the mouse-protection test and is clearly a matter which must be resolved.

As to the role of adjuvant, there are at least two well documented reports showing that the commonly used aluminium hydroxide has little or no effect on the immune response in mice (Table II) [30, 31].

Acknowledgement of the failure of adjuvant to enhance the protective response is supported by the fact that both adsorbed and non-adsorbed vaccines are assayed by control authorities against a non-adsorbed reference preparation. For this to be possible the slopes of the dose-response curves of both type of preparation, adsorbed and non-adsorbed, must be acceptably parallel. When adjuvant enhances the protective response, as with tetanus toxoid, the responses are usually non-parallel. Should then agglutinin-responses, which are markedly higher with adjuvant vaccines, be rejected as an alternative to the mouse-protection test because they indicate a host-response not indicated by the latter test or should the mouse-protection test be re-assessed? Is it possible that the correlation thought to have been established between protection in mice and in children in the definitive M. R. C. trials [26] could have been fortuitous?

So far a number of unresolved problems in the metabolism of pertussis and in the standardization of vaccines have been indicated. Another area which causes concern is that of reactions to vaccine. Perhaps it is not too surprising that such should be the case when our understanding of the micro-organism from which the vaccine is made is so incomplete. Many unwarranted assumptions and assertions are made in the debate on this topic. It seems to be accepted, for instance, that regardless of source, pertussis vaccines are identical, that there is indeed a single entity identifiable as pertussis vaccine just as there is only one sodium chloride. Nothing could be further from the truth, as can be readily noted from the table indicating the methods of manufacture of the different vaccines used in the M. R. C. trial [26]. Moreover, of

Table II

Comparison between the potency of the pertussis component of 10 lots of plain (non-adsorbed) and adsorbed D.T.P. vaccine

Parent pertussis component	Plain (non-adsorbed) D.T.P. vaccine		Adsorbed D.T.P. vaccine	
	Potency (P.U. per dose)	Potency ratio*	Potency (P.U. per dose)	Potency ratio*
15.2			5.7	0.38
15.08			2.6	0.17
31.0			3.6	0.12
			8.1	0.26
4.3	6.1	1.42	5.1	1.19
4.2	4.2	1.00	2.1	0.50
3.9			2.8	0.72
			3.2	0.82
4.5	5.9	1.31	2.5	0.56
6.9	8.4	1.22		
	13.0	1.88		
9.2	7.1	0.77		
	4.9	0.53		
5.7	3.8	0.67	14.5	2.54
6.1	4.7	0.77		
	5.0	0.82		

Plain D. T. P. Adsorbed D. T. P. Difference

Arithmetic Mean Potency 1.04 0.73 Not significant

Geometric Mean Potency 0.92 0.52 $p = 0.05$

* The potency ratio is obtained as: $\left\{ \begin{array}{l} \text{Potency of Plain or Adsorbed D.T.P. Vaccine} \\ \text{Potency of parent pertussis component} \end{array} \right.$

the known properties of the micro-organism more are not measured than are measured and the method of manufacture is not even taken into account.

Pertussis vaccines from different manufacturers can only be similar — not necessarily identical — in respect of the properties measured — sterility, safety, mouse-toxicity, potency. As far as mouse-toxicity, histamine-sensitizing activity and lymphocytosis-promoting activity are concerned there may be even greater differences in absolute terms than are imagined because of our failure to develop reference preparations for their assay. Of significance in this respect is the report by STAINER and SCHOLTE [32] of their ability to vary the amounts of heat-labile toxin, histamine-sensitizing factor and mouse-protect-

tive antigen produced by *B. pertussis* in STAINER and SCHOLTE's medium [5] by varying the amounts of glutamic acid and proline in the medium. On the other hand, another series of experiments carried out in connection with the development of this medium was aimed at determining the effect of passage on the ability of strains to produce protective antigen. Strains were passaged serially, twice weekly, for 50 passages and potency assays carried out at intervals of 10 passages. No significant decrease in ability to produce protective antigen was noted. The mouse-protective antigen, the basis of all pertussis vaccines, is one antigen produced by the micro-organism on which there seems to be no literature regarding the ability to control its production either by changing the composition of growth medium or by selecting strains although CAMERON [15] indicated that it may be possible to obtain greater yields of protective antigen by cloning.

Today some vaccines are killed with heat, some with formalin, some with merthiolate, some with combinations of all three with particular emphasis on temperature, time and concentration relationships. Some vaccines are grown in fermenters, some on solid surfaces. The work of PUSZTAI *et al.* [33] and of VAN RAMSHORST [34] shows quite clearly that when acid-precipitation is used to harvest micro-organisms from fermenters a more toxic vaccine results. Do all strains of *B. pertussis* produce this toxic factor? If it is not produced by strains grown on solid surfaces, do cells so produced possibly differ in other ways from those produced in fermenters?

Are these points important? Presumably they must be wherever the vaccine is the centre of controversy and to be consistent in our thinking, if pertussis vaccine continues to be regarded as potentially dangerous we are being remiss in our responsibility in not taking these other factors into account.

REFERENCES

1. BORDET, J., GENCOU, O.: Ann. Inst. Pasteur **20**, 731 (1906).
2. GOLDNER, M., JAKUS, C. M., RHODES, H. K., WILSON, R. J.: J. gen. Microbiol. **44**, 439 (1966).
3. VAJDIC, A. H., GOLDNER, M., WILSON, R. J.: J. gen. Microbiol. **44**, 445 (1966).
4. STAINER, D. W.: Symp. Serological and immunobiological Standardization **13**, 89 (1970).
5. STAINER, D. W., SCHOLTE, M. J.: J. gen. Microbiol. **63**, 211 (1970).
6. PROOM, H.: J. gen. Microbiol. **12**, 63 (1955).
7. ROWATT, E.: J. gen. Microbiol. **17**, 279 (1957).
8. VERWEY, W. F., THIELE, E. H., SAGE, D. N., SCHUCHARDT, L. F.: J. Bact. **58**, 127 (1949).
9. MAZLOUM, H. A., ROWLEY, D.: J. Path. Bact. **70**, 439 (1955).
10. CAMERON, J., DUNNING, J. M.: Int. Congr. Microbiology, Mexico 1970.
11. ENSMINGER, P. W., CULBERTSON, C. G., POWELL, H. M.: J. infect. Dis. **93**, 266 (1953).
12. COHEN, S. M., WHEELER, M. W.: Amer. J. publ. Hlth **36**, 371 (1946).
13. POLLOCK, M. R.: Brit. J. exp. Path. **28**, 295 (1947).
14. JEBB, W. H. H., TOMLINSON, A. H.: J. gen. Microbiol. **13**, 1 (1955).
15. CAMERON, J.: J. Path. Bact. **94**, 367 (1967).
16. STANBRIDGE, I. N., PRESTON, N. W.: J. Hyg. (Lond.) **73**, 305 (1974).
17. CAMERON, J.: Progr. immunobiol. Stand. **3**, 319 (1969).
18. EVANS, D. G., PERKINS, F. T.: Brit. J. exp. Path. **35**, 322 (1954).

19. EVANS, D. G., PERKINS, F. T.: Brit. J. exp. Path. **36**, 391 (1955).
20. ANDERSEN, E. K.: Acta path. microbiol. scand. **40**, 227 (1957).
21. EVANS, D. G., PERKINS, F. T.: J. Path. Bact. **66**, 479 (1953).
22. EVANS, D. G., PERKINS, F. T.: J. Path. Bact. **68**, 251 (1954).
23. PILLEMER, L., BLUM, L., LEPOW, I. H.: Lancet **1**, 1257 (1954).
24. Report: Brit. med. J. **1**, 1463 (1951).
25. Report: Brit. med. J. **2**, 454 (1956).
26. Report: Brit. med. J. **1**, 994 (1959).
27. PRESTON, N. W.: J. Path. Bact. **91**, 173 (1966).
28. BUTLER, N. R., VOYCE, M. A., BURLAND, W. L., HILTON, M. L.: Brit. med. J. **1**, 663 (1969).
29. PRESTON, N. W., MACKAY, R. I., BAMFORD, F. N., CROFTS, J. E., BURLAND, W. L.: J. Hyg. (Lond.) **73**, 119 (1974).
30. CAMERON, J.: Proc. Symp. on Combined Vaccines, Yugoslav AC SC and Arts Zagreb, 1972. pp. 55-67.
31. NOVOTNY, P., BROOKES, J. E.: J. biol. Stand. **3**, 11 (1975).
32. STAINER, D. W., SCHOLTE, M. J.: Bact. Proc. p. 100 (1969).
33. PUSZTAI, Z., JOÓ, I., JUHÁSZ, V. P.: Z. Immun.-Forsch. **122**, 278 (1961).
34. VAN RAMSHORST, J. D.: Progr. immunobiol. Stand. **3**, 324 (1969).

Address of the authors:

JACK CAMERON, DENNIS W. STAINER
Connaught Laboratories Limited
1755 Steeles Avenue West, Willowdale, Ontario M2N 5T8 Canada

MORPHOLOGY OF SALMONELLA TYPHI IN DIFFERENT INACTIVATION TECHNIQUES

P. BERGMANN, SONJA MEBEL and SIGLINDE RUSTENBACH

State Institute for Immune Preparations and Culture Media, Berlin, G.D.R.

(Received September 16, 1976)

Heat-phenol treatment of *Salmonella typhi* results in a denaturation of the cytoplasm and a smoothing of the cell wall with numerous ruptures. Following acetone inactivation the cell wall changes similarly, the cytoplasm is shrunken and its structures remain intact. Destruction is more intensive following heat-phenol treatment than after acetone precipitation. In both cases three contrasting layers of the cell wall can be seen.

Antityphoid vaccination is one of the oldest bacterial vaccinations. Parenteral vaccination is accompanied by considerable reactions [1, 2]. Various methods are being employed for the inactivation of bacterial cells. Heat-phenol and acetone are much in use while inactivation by alcohol, formaldehyde-phenol and chloroform plays a subordinate role.

A number of some comparative field trials of heat-phenol (L-vaccine) and acetone vaccine (K-vaccine) carried out by the WHO showed that both vaccines are of a high reactogenicity and are very effective with the K-vaccine being more effective [3, 4]. Doubtlessly, the various inactivation methods cause different changes of the cell. The aim of the present study was to examine how inactivation by heat and preservation by phenol or treatment with acetone influences the morphology of the bacterial cell. Conclusions as to effectivity and reactogenicity of the different vaccines have been avoided.

Material and methods

Strains. Salmonella typhi Ty2.

Culturing conditions. As a nitrogen source for all culture media a casein-hydrolysate of our own production is used. Cultivation on solid medium is done in Roux flasks which are inoculated with a six-hour shaken culture. Harvesting follows after six-hour incubation at 37°C. The cells are suspended in saline. The bacterial suspension obtained is processed further immediately. Cultivation on liquid medium is done in 20 litre flasks inoculated with a six-hour shaken culture. Inoculation density is 5×10^8 cells per ml. If aerated intensively, the culture is centrifuged after incubation at 37°C for 18 hr (Cepa-centrifuge, 10 000 g). The cells resuspended in saline are processed further immediately.

Inactivation of bacteria. (a) Bacterial suspensions obtained from solid or liquid media adjusted with saline to 10 International Opacity Units (IOU) serve as control. Immediately thereafter the bacteria are prepared for electron microscopy. (b) For heat-inactivation the suspension is adjusted to 20 IOU. Determination of bacterial density is done in Specol (Zeiss, Jena) at 530 nm. This suspension is incubated in a water-bath one hour each at 56°C on three days. (c) Phenol-inactivation: bacterial content as indicated under (b); 0.5% phenol is added

to the suspension. (d) Heat-phenol inactivation: bacterial content and heat treatment as under (b), and the suspension is mixed with phenol as under (c). (e) Phenol-heat inactivation: bacterial density as under (b), phenol admixture as under (c), heat treatment as under (b). (f) Acetone precipitation: bacteria obtained from liquid media are adjusted to 20 IOU and acetone precipitation is done as described in reference [5]. (g) Lyophilization. Samples of the bacterial suspensions treated as under (a) and of the bacteria treated as indicated under (f) were lyophilized. The substance used contains gelatin and sucrose and is added to a suspension of 20 IOU per ml in the proportion of 1 : 2, so that the final concentration is 10 IOU per ml. Before preparing them for electron microscopy, all bacterial cultures are adjusted with saline to a bacterial density of 10 IOU per ml.

Electron microscopy. The bacterial cells are washed with physiological saline solution and prefixed for two hours in 2% glutaraldehyde in phosphate buffer according to Sørensen (0.05 M, pH 7.4), then washed in 0.18 M saccharose in Sørensen buffer (0.08 M, pH 7.4) and post-fixed for two hours with 1.5% osmium tetroxide in veronal-acetone buffer (0.12 M, pH 7.4). The fixed cells are dehydrated in aqueous ethanol (25%, 50%, 70%, 96%) followed by absolute ethanol and embedded in Epon 812 after infiltration with propylene oxide. Ultrathin sections cut with an LKB Ultratome III are stained with 3% uranyl acetate in 50% aqueous ethanol. Observations were made with a KEM 1-2 electron microscope (VEB Werk für Fernsehelektronik, G.D.R.) and a JEM 100 B electron microscope (Jeol, Japan).

Results

(a) *Control.* Untreated cells show the normal structure of *S. typhi*. The multi-layered structure of the cell wall is clearly visible showing the typical, wavy profile. The cytoplasm contains nucleoids and is interspersed with many ribosomes. The cytoplasmic membrane is indistinct. Cultivation of cells on agar leaves some flagella in contrast to cultivation in liquid media (Figs 1-3).



Fig. 1. *S. typhi* cultivated on agar. Flagella are visible. $\times 28\,000$

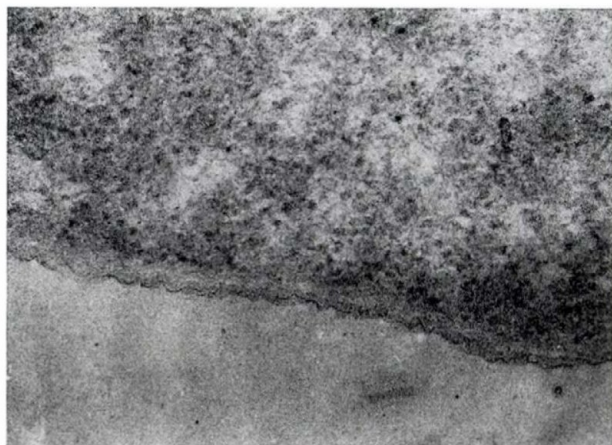


Fig. 2. *S. typhi*. Same culture as in Fig. 1. $\times 90\ 000$

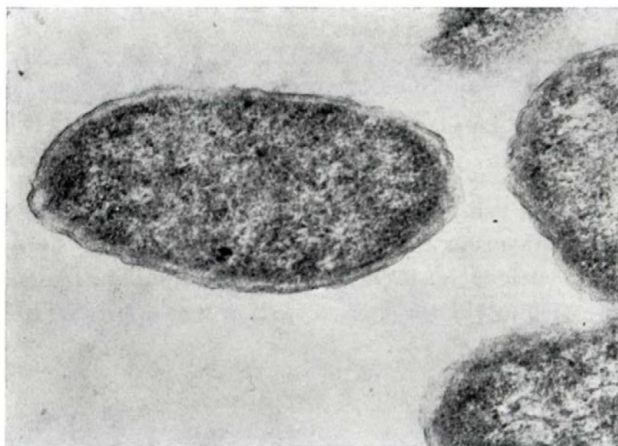


Fig. 3. *S. typhi* cultivated at 37°C in liquid shake culture. Flagella are not visible. $\times 60\ 000$

(b) *Heat-inactivation*. Heat treatment of the cell generally stretches and smoothes the cell wall; its layers lie parallel. The cytoplasm is destroyed and forms contrasting areas concentrated at the cytoplasmic membrane but also in the interior of the cell. The space adjacent to the contrasting areas is unstained. The cytoplasmic membrane is often deformed into the inner of the cell with an expansion of the periplasmic space. In a few cases the cell wall was deformed or broken (Figs 4, 5).

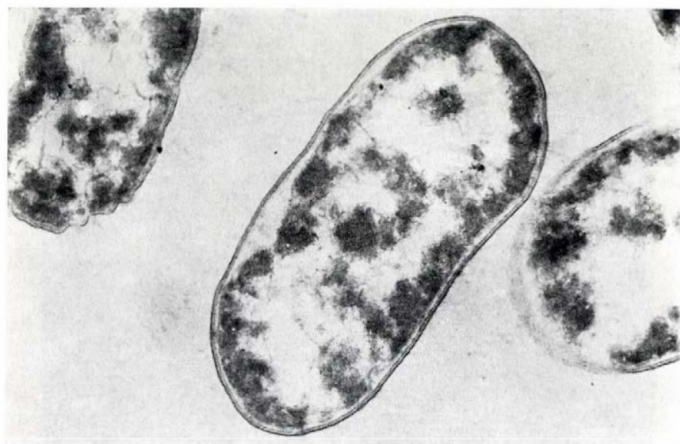


Fig. 4. *S. typhi* heat-killed cells. The cytoplasm is denatured and aggregated forming electron-dense clumps. Note the smooth cell wall. $\times 50\ 000$



Fig. 5. *S. typhi*. Details of the wall of heat-treated cell. $\times 150\ 000$

When cultivating cells in liquid media the cell wall is less stretched and the cytoplasm is denatured. Single, strongly contrasting sites in unstained areas are connected with each other in a thread-like manner and some diffusely contrasting areas without distinct structure are visible (Fig. 6).

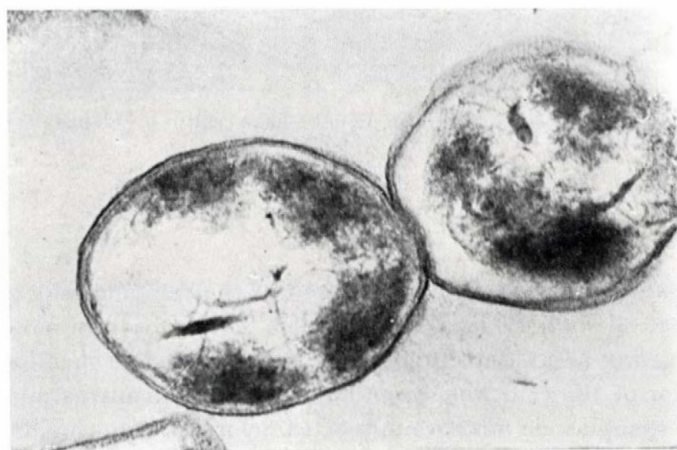


Fig. 6. *S. typhi* liquid medium culture treated as in Figs 4-5. Aggregation of the cytoplasm is less distinct. $\times 74\ 000$

(c) *Phenol-inactivation*. Phenol has less influence on the morphology of the bacterial cell than heat treatment. Compared to the control, the cell appears more stretched and smoother. Nucleoids such as ribosomal structures are discernible, though compared with the untreated cell the cytoplasm is less contrasting. In a few cases the cytoplasm is denatured. The effect of phenol is especially obvious when the cells are cultivated on solid media, whereas the cells cultivated in liquid media are hardly altered (Figs 7, 8).

(d) *Heat-phenol inactivation*. Inactivation of agar cultures of *S. typhi* by heat and preservation with phenol led in all observed cases to a completely altered appearance of the cytoplasm and the cell wall. In the cell opaque areas

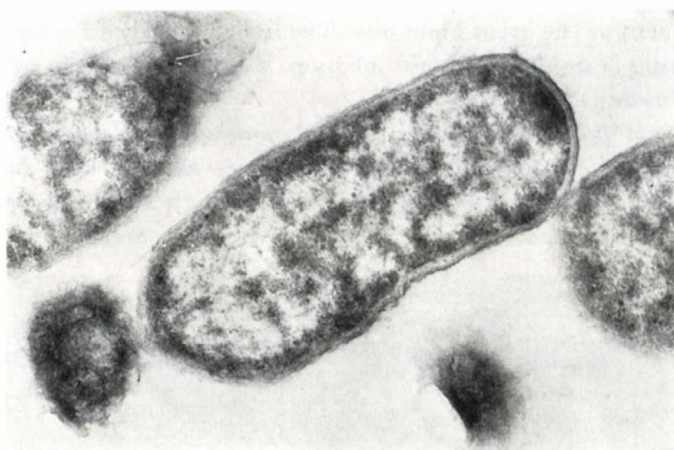


Fig. 7. *S. typhi* phenol-treated cell. The wall is stretched, but less than with heat treatment.
× 42 000



Fig. 8. *S. typhi* liquid medium culture treated as in Fig. 7. The influence of phenol is less distinct.
× 63 000

are seen, mostly near the cytoplasmic membrane, and large areas remain electron optically translucent. The cytoplasm is denatured and coarsely clumped, nucleoids and ribosomes are no longer visible. In many cells the cytoplasmic membrane is intact but sometimes aggregated, the membrane is folded and is partly disintegrating into clearly visible small fragments. The cell wall is not wavy but altogether very distinct and stretched with three electron optically dense parallel layers. The R-layer and the outer layer are well contrasted while the middle one is not very distinct. The wall is about 15 nm thick. Especially characteristic of the heat-phenol-killed cells is a breaking up of the cell wall with typical deformations. The cell wall is ballooned, forms small vesicles and then ruptures (Figs 9–11). The vesicles are smaller and less frequent as the great blebs observed in heat-treated *Escherichia coli* [6]. When culturing is done in a liquid medium, destruction of the cytoplasm and of the membrane is less complete.

(c) *Phenol-heat inactivation.* Phenol and subsequent heat treatment result in nearly the same morphological changes as does heat-phenol inactivation. The cell wall becomes smooth and the single layers are parallel. The cytoplasm is denatured, forming dense, contrasting areas in the inner cell

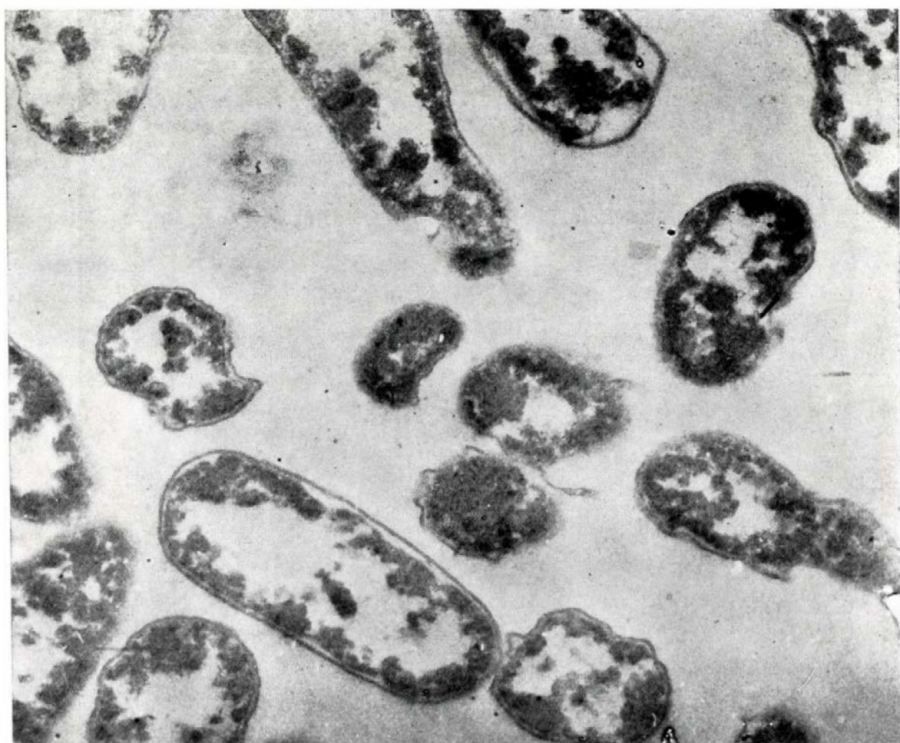


Fig. 9. *S. typhi* heat-phenol-killed cell. The cytoplasm is denatured and aggregated. The cell wall is smoothed or broken after small ballooning. $\times 28\ 000$



Fig. 10. *S. typhi* treated as in Fig. 9. Note the five-layered cell wall with three electron-dense lines. $\times 90\,000$

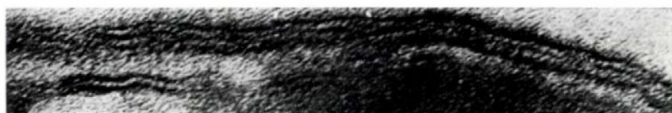


Fig. 11. Detail of Fig. 10 at higher magnification. $\times 250\,000$

which is translucent. The degree of denaturation seems slightly lower than after heat-phenol inactivation. Cells from liquid media are morphologically less changed (Figs 12, 13).

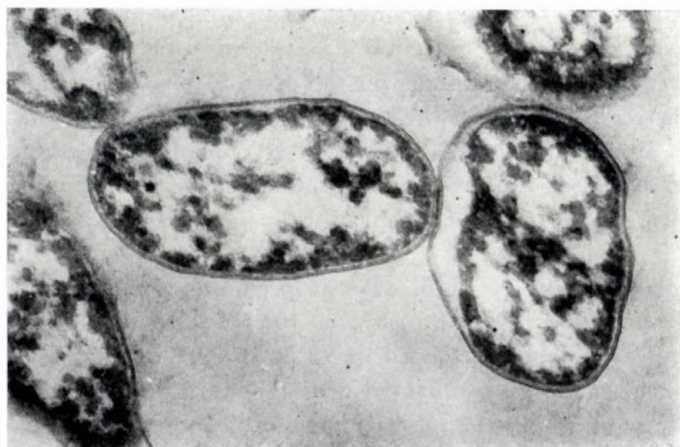


Fig. 12. *S. typhi* phenol-heat-killed cell. The morphology of the cell is similar as after heat-phenol inactivation. $\times 38\,000$

(f) *Acetone precipitation.* Acetone precipitation causes considerable changes. Compared with the control the whole cellular space is bright and the cytoplasm is mostly intact. The nucleoids form thin agglomerates and, compared to heat-phenol treatment, the ribosomal units are clearly visible. Only



Fig. 13. *S. typhi* liquid medium culture treated as in Fig. 12. Destruction of the cytoplasm is less distinct. $\times 60\ 000$

in a few cases are less dense areas observed in the cytoplasm. Despite the sharp separation of the cytoplasm from the periplasmic space, the cytoplasmic membrane is usually unobservable. A contracted cytoplasm and thus an extended periplasmic space is typical of acetone inactivation. The cell wall is smooth, clearly divided into three contrasting layers and two light ones and shows a well-contrasted distinct profile. The thickness of the cell wall is about 15 nm as in (a). Breaks in the cell wall are less frequent than after heat-phenol treatment. A bending of the cell wall and a localized blurring of its layers in the otherwise distinctly contoured cell membrane was observed (Figs 14–16).

(g) *Lyophilization.* Lyophilization of untreated cells (a) causes shrinking. The diameter decreases from 0.80 μm to 0.40 μm on the average. The cell wall is more wavy. The elementary structures of the cell remain intact (Fig. 17). Freeze-drying of acetone-precipitated cells (f) cause a shrinkage of the whole cell as in the case of untreated salmonellae. There is an obvious contrast intensification of the three electron-dense layers while the structure of the cytoplasm remains unchanged (Figs 18, 19).

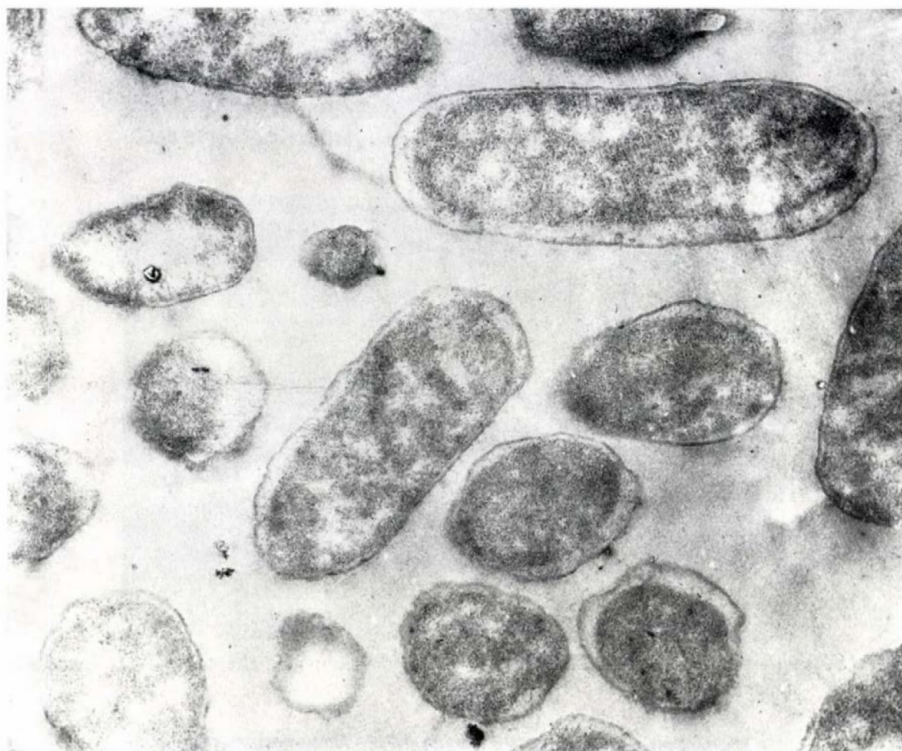


Fig. 14. *S. typhi* acetone-treated cell. The entire cytoplasm is contracted and separated by the expanded transparent periplasmic space from the five-layered cell wall. $\times 28\ 000$

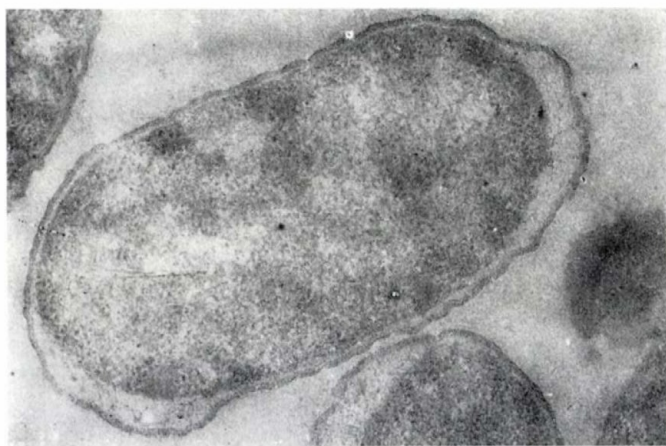


Fig. 15. *S. typhi* treated as in Fig. 14. $\times 54\ 000$

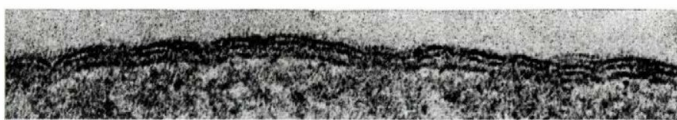


Fig. 16. Detail of Fig. 15 at higher magnification. $\times 150\,000$

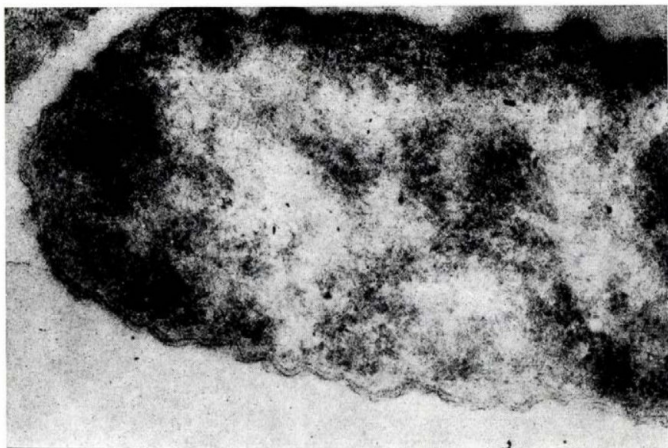


Fig. 17. *S. typhi* lyophilized cell. The entire cell is shrunken and the cell wall is more wavy. $\times 72\,000$

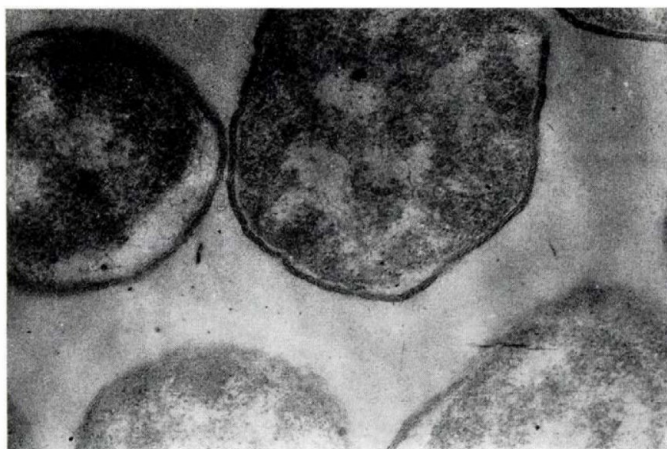


Fig. 18. *S. typhi* acetone-treated cells after lyophilization. The cells are shrunken. The cell wall is smooth, five-layered and more electron-dense than in Figs 14–16. $\times 54\,000$

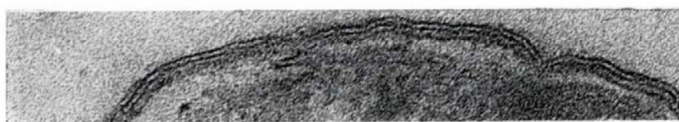


Fig. 19. Detail of Fig. 18 at higher magnification. $\times 150\,000$

Table I

Effect of different inactivation techniques on the morphology of S. typhi

Method of inactivation and/or treatment	Figure	Cell wall	Nucleoid	Cytoplasm
a	1-3	waved, three-layered	fibrinous	granulous
b	4-6	stretched, five-layered	denatured	denatured
c	7-8	slightly stretched	fine agglomerates	less shrunken
d	9-11	stretched, five-layered, small bulges, frequently broken	denatured	denatured
e	12-13	stretched, five-layered, small bulges, frequently broken	denatured	denatured
f	14-16	smooth, five-layered frequently bent	slightly denatured	slightly denatured
g (control)	17	fairly waved shrunken	unchanged	unchanged
g	18-19	shrunken contrast-intensification	slightly denatured	slightly denatured

Discussion

As a result of heat inactivation of bacterial cells, breaks of the nuclear DNA, a degradation of ribosomes, denaturation of proteins and changes of the membrane occur [7, 8]. The influence of organic substances is less known.

SLONIM *et al.* [9] claim that under the effect of phenol at a concentration of 1%, surface lipids are removed. The acetone inactivated K-vaccine contains fewer organic substances than the heat-phenol inactivated L-vaccine [10].

A comparison between heat-phenol inactivated bacterial cells of *S. typhi* Ty2 and those inactivated by acetone shows a particularly heavy destruction of cellular elements. The cytoplasm and the cellular organs such as nucleoids and ribosomes are denatured by heat and form osmiophilic dense agglomerates. Parts of the cytoplasmic membrane are carried into the inner cell and the space free from agglomerates remains unstained. The effect of heat often causes ruptures of the cell wall. Exposure to 100°C is a well-known method for isolating the cell wall of Gram-negative bacteria with a coagulated protoplast in small yields [11]. But even when a temperature of 55°C is applied the cellular proteins are denatured, the cell wall ruptures, the cytoplasm breaks through the cell wall and the DNA changes [6]. The first effect of heat treatment during heat-phenol inactivation is seen on the membrane. It is stretched and

smoothed, clearly visible and frequently broken. A distinct, five-layered structure of the cell wall is visible with three electron-dense layers with well contrasting basal and outer layers. The middle, dense layer is less distinct and flanked by two light, electron-transparent layers. Similar changes of the cytoplasm and a smoothing of the cell wall have been observed in *E. coli* [12]. Denaturation of membrane proteins and cytoplasm are primarily caused by heat. Probably the whole cell wall is stiffened and its flexibility is impaired due to the denaturation of membrane proteins.

The membranes are stretched, the cytoplasm coagulates and forms electron-dense clumps adhering mainly to the cytoplasmic membrane. If the cells are treated with phenol only, the membrane remains normal. The DNA is seen as a threadlike structure, the cytoplasm and the organelles remain intact. Even a change of the phases of treatment (phenol, then heat) results mainly in the same morphology as caused by heat-phenol inactivation. Acetone treatment causes characteristic changes of the cell wall too, but the membrane is less smooth than after heat-phenol inactivation. The five-layered structure of the cell wall is more distinct and all three electron-dense layers are well contrasting.

The membrane proteins are denatured by acetone and the lipid parts and other acetone-soluble substances of the cell wall are probably removed. Acetone penetrates through the cell wall and contracts the whole cytoplasm without essentially destroying the cytoplasmic membrane, the cytoplasm and its structures. Ruptures of the cell wall are rare, but the cell wall frequently stretches into the periplasmatic space.

In some cell wall areas the effect of acetone is so strong that the borders between the three otherwise well-contrasting layers become blurred.

The cellular structure of *S. typhi* is considerably changed after both heat-phenol treatment and acetone inactivation. The morphology, however, changes differently as a result of various treatments. Thus, heat treatment leads to a specially distinct denaturation and to breaks in the cell wall.

It must be stressed that no conclusions can be drawn from the described changes concerning the effectiveness and reactogenicity of typhoid vaccines.

Acknowledgements. We are indebted to Professor A. A. AWAKYAN and his co-workers, Gamaleya-Institute, Moscow, U.S.S.R., for providing the slides from JEM 100 B as well as for stimulating discussions and useful advice; and to Miss JUTTA NEBEL and Mrs. EDITH KANT for technical co-operation.

REFERENCES

1. Joó, I.: Ann. immunol. hung. **15**, 7 (1971).
2. NEBEL, S., RUSTENBACH, S.: Z. ärztl. Fortbild. **67**, 17 (1972).
3. Typhoid Panel, UK Department of Technical Cooperation: Bull. Wld Hlth. Org. **30**, 631 (1964).
4. Yugoslav Typhoid Commission: Bull. Wld Hlth Org. **26**, 357 (1962).
5. Walter Reed Army Institute of Research: Bull. Wld Hlth Org. **30**, 635 (1964).

6. SCHEIE, P., EHRENSPECK, S.: J. Bact. **114**, 814 (1973).
7. RUSSEL, A. D., HARRIES, D.: Appl. Mikrobiol. **15**, 407 (1967).
8. SEDGWICK, S. G., BRIDGES, B. A.: J. gen. Microbiol. **71**, 191 (1972).
9. SLONIM, D., DIAWARA, S. M., BRAZDOVA, R.: J. Hyg. Epid. (Praha) **13**, 313 (1969).
10. Walter Reed Army Institute of Research and International Laboratory for Biological Standards, Statens Serum Institut: Bull. Wld Hlth Org. **30**, 647 (1964).
11. SALTON, M. R. J.: The Bacterial Cell Wall. Elsevier Publ. Co., Amsterdam 1964.
12. DE PETRIS, S. J.: Ultrastruct. Res. **19**, 45 (1967).

Address of the authors:

P. BERGMANN, SONJA MEBEL, SIGLINDE RUSTENBACH
State Institute for Immune Preparations and Culture Media
Klement-Gottwald-Allee 317/321, DDR-112 Berlin, German Democratic Republic

PATHOGENIC EFFECT OF ENTEROTOXIGENIC ESCHERICHIA COLI AND ESCHERICHIA COLI CAUSING INFANTILE DIARRHOEA*

YU. E. POLOTSKY, E. M. DRAGUNSKAYA, V. G. SELIVERSTOVA,
T. A. AVDEEVA, M. G. CHAKHUTINSKAYA, I. KÉTYI, A. VERTÉNYI,
B. RALOVICH, L. EMŐDY, I. MÁLOVICS, N. V. SAFONOVA,
E. S. SNIGIREVSKAYA and E. I. KARYAGINA

Department of Pathological Anatomy, Institute of Experimental Medicine, USSR
Academy of Medical Sciences; Department of Enteric Infections, Leningrad
Pasteur Institute of Epidemiology and Microbiology, Leningrad, Institute of Microbiology,
University Medical School, Pécs, Hungary and Institute of Cytology, U.S.S.R.
Academy of Sciences, Leningrad, U.S.S.R.

(Received October 27, 1976)

Macroscopic, light and electron microscopic alterations in ligated rabbit intestinal loops challenged with five standard enterotoxigenic *Escherichia coli* (ETEC) and twenty-three enteropathogenic *E. coli* (EEC-I) strains, freshly isolated from infantile enteritis cases, were investigated. Only two O26 : K60 : H11 strains produced enterotoxin. Their living cultures, sterile filtrates of the fluid medium and ultrasonic lysates of the bacteria resulted in pronounced hypersecretion of the intestinal epithelium followed by fluid accumulation and loop dilatation. These two *E. coli* strains, similarly as the other loop-negative EEC-I strains, were able to penetrate into the intestinal epithelium. In contrast to the standard ETEC strains, the EEC-I bacteria, adhering to the brush border, intruded into the microvilli, multiplied on the outer epithelial cell membrane making close contact with it and, causing shedding of microvilli, penetrated into enterocytes becoming enclosed in membrane-bound phagosome-like vacuoles, appeared in the lamina propria and elicited mild focal polymorphonuclear infiltration.

Enteropathogenic *Escherichia coli* (EEC) may cause different enteric infections in human beings. Serotypes which are involved in infantile enteritis have been called EEC of the first category (EEC-I). Pathogens of dysentery-like diseases are regarded shigella-like EEC strains belonging to the second category (EEC-II). The enterotoxigenic *E. coli* (ETEC) strains produce cholera-like disease in adults and children and show some resemblance to the agents of enterotoxic coli-bacillosis of animals [1-5]. Earlier we have studied some of the EEC-I and ETEC strains and compared their properties with those of other enteropathogens [6-10]. In this paper we present results proving the capacity of certain EEC-I strains to produce enterotoxin.

Materials and methods

Bacterial strains are listed in Table I. The five standard ETEC strains were kindly supplied by Drs H. L. DUPONT, S. L. GORBACH and H. W. SMITH. Purified cholera toxin (lot 4493G) was prepared under contract for the National Institute of Allergy and Infectious Diseases

* Dedicated to the 70th anniversary of Professor M. V. VOINO-YASENETSKY, Head of the Department of Pathological Anatomy, Institute of Experimental Medicine, U.S.S.R. Academy of Medical Sciences, Leningrad.

(NIAID), National Institute of Health, Bethesda, Maryland, U.S.A. and originated from Dr. R. A. FINKELSTEIN. EEC-I strains were freshly isolated from infantile enteritis cases in Pécs, Budapest and Leningrad.

Table I
List of strains

Designation	Serotype	Origin	References
B2C	O6 : - : H16	DuPont, H. L.	[3]
B7A	O148 : - : H28		
339T5	O15 : - : H11	Gorbach, S. L.	[11, 12]
410 G	O78 : - : H12		
P105	O138 : K81	Smith, H. W.	[13]
1, 2	O26 : K60 : H-	Pécs, routine material	
3, 4, 5	O26 : K60 : H11		
6	O26 : K60 : H?		
7, 8	O55 : K59 : H4		
9	O55 : K59 : H?		
E-5	O78 : K80 : H?	Czirók, É.	[14]
10, 11	O111 : K58 : H2	Pécs, routine material	
548, 8910	O111 : K58 : H2	Geck, P., Budapest	[15]
1062, 1065, 1066	O111 : K58 : H-	Pécs, routine material	
12	O111 : K58 : H?	Rédey, B., Veszprém	
56899	O114 : K90 : H-	Czirók, É.	
438, 439, 466, Kr.	O4 : - : H5	Pécs, routine material	

Media, cultivation and preparation of the materials. The strains were grown in meat-peptone broth (MPB) or in modified medium of SAKAZAKI *et al.* [16] with shaking at 37°C for 16–24 hr. Supernatants (S) were harvested from 18–24 hr shaken cultures, centrifuged at 3000–4000 g and filtered through Millipore filter 0.45 µm. Whole cell lysate (WCL) was produced from the same cultures. The sediment of the cultures was destroyed in ultrasonic desintegrator under cooling, at 18–20 KHz, for 30 min, and the suspension was filtered through a membrane filter [17].

Gut loop test was performed in rabbits weighing 1600–3600 g, according to the method of TAYLOR *et al.* [18] with slight modification [7]. Evaluation of the test was carried out under intravenous anaesthesia 2 and 3 hr after challenge but mostly 6–10, 13–15 or 17–18 hr after the injection. Occasionally the experiment was prolonged to 24 hr. Visible results were recorded and the fluid content of the loops was measured. Viable germ count of the fluid was also determined by serial dilution and spreading onto nutrient agar plates. After emptying of the loops their length was measured and the ratio of the fluid volume per the length of the loop was calculated (positive result, >0.5).

Suckling-mouse test [19] for detection of heat-stable (ST) enterotoxin was performed according to the modification of JACKS and WU [20].

Intradermal test to demonstrate the vascular permeability factor (PF) was carried out by the method of EVANS *et al.* [21], injecting intravenously 0.12 ml/100 g body weight of 5% Pontamine Sky Blue 6BX (Serva, Heidelberg) into rabbits 18 hr after intracutaneous inoculation of enterotoxin. Readings were made 1 hr after intravenous injection.

Microscopic examinations. For light microscopy, the emptied loops were immediately placed on thick paper labels, cut longitudinally, fixed in 10% formalin, and embedded in paraffin and gelatin. Frozen gelatin sections were stained with Goldmann's sudan- α -naphthol to select granular leukocytes. Paraffin sections were stained with eosin-azure, thionin, Leishman's, Dominici's and Hotchkiss's dyes.

For electron microscopy, small pieces were cut out from some loops after opening the abdominal cavity and recording gross results. The specimens were fixed in chilled 2.5% glutaraldehyde in phosphatase or cacodylate buffer pH 7.2-7.4 with addition of thiophosphamide or lanthanum to reveal the glycocalyx [7, 8]. Thereafter the specimens were postfixed in 1% osmium tetroxide and embedded in Araldite. Semithin, 1 μ m thick, and ultrathin sections were cut with LKB and Reichert ultratomes. The 1 μ m sections were stained with toluidine blue and azure, and examined with light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined by EMV-100L and JEM-7 electron microscopes operating at 75 and 80 Kv.

Results

The standard ETEC strains, their S and WCL preparations, except 410 G (O78 : H12) strain, gave in most cases positive results in the intestinal loops 6 hr after challenge, but sometimes earlier. The effect of cholera toxin (18 Lb/ml) was the same. All the S and WCL preparations were positive in the suckling mouse-test and intradermal (PF) test (Table II).

Table II
Enterotoxin production by standard ETEC strains

Strain and serotype	Loop test				Suckling mouse test bowel/body weight ratio		Intradermal test
	number of loops		fluid volume/length of loop*				
	tested	positive	range	average			
1. Living cultures:							
B2C (O6 : H16)	27	24	0.26-2.59	1.36			
339T5 (O15 : H11)	14	13	0.41-2.89	1.85			
410G (O78 : H12)	13	2	0.0-0.50	0.17			
B7A (O148 : H28)	33	33	0.82-2.89	1.79			
P105 (O138 : K81)	12	9	0.0-1.20	0.80			
2. Supernatants (S):							
B2C (O6 : H16)	7	7	0.84-2.50	1.61	0.10	positive	positive
339T5 (O15 : H11)	5	4	0.25-2.38	1.50	0.09	positive	positive
410G (O78 : H12)	9	2	0.0-0.86	0.25	0.09	positive	positive
B7A (O148 : H28)	21	21	1.10-3.08	1.60	0.12	positive	positive
P105 (O138 : K81)	15	13	0.0-1.50	0.88	0.13	positive	positive
3. Whole cell lysates (WCL):							
B2C (O6 : H16)	5	5	1.43-2.00	1.66			positive
339T5 (O15 : H11)	2	2	0.60-0.70	0.65			positive
410G (O78 : H12)	7	3	0.0-1.73	0.52			positive
B7A (O148 : H28)	3	3	1.07-1.88	1.36			positive
Cholera ⁿ Lot 4493	3	3	1.07-1.33	1.16			positive
Meat-peptone broth (MPB)	37	1	0.0-0.50	0.07			not tested**

* Results are negative when the ratio is less than 0.5.

** SAKAZAKI's medium and diluent PBS served as negative control material

In contrast, only two (strains Nos 3 and 4, both of O26 : K60 : H11 serotype) out of the EEC-I strains resulted in fluid accumulation and loop dilatation; their S and WCL preparations were also positive (Table III). The filtrates of these two strains (Nos 3, 4) and two others (E-5 and 56 899) were tested by the rabbit intradermal (PF) assay. The reaction was strongly positive with strains Nos 3 and 4, slightly positive with strain No. 56 899, and consistently negative with strain E-5. Germ count of the fluid from the dilated

Table III

Loop test with EEC-I strains, their supernatants (S) whole cell lysates (WCL)

Strain and serotype	Number of loops		Fluid volume/length of loop	
	tested	positive	range	average
<i>1. Living cultures:</i>				
1 O26 : K60 : H-	2	0	0.0-0.15	0.08
2 O26 : K60 : H-	2	0	0.0-0.15	0.10
3 O26 : K60 : H11	30	24	0.10-1.57	0.75
4 O26 : K60 : H11	19	12	0.04-1.57	0.68
5 O26 : K60 : H11	2	0	0.0	0.0
6 O26 : K60 : H4	4	0	0.0	0.0
7 O55 : K59 : H4	3	0	0.15-0.25	0.20
8 O55 : K59 : H7	2	0	0.0	0.0
9 O55 : K59 : H.	4	0	0.0	0.0
E-5 O78 : K80 : H-	10	0	0.0-0.39	0.07
10 O111 : K58 : H2	6	0	0.0-0.20	0.05
11 O111 : K58 : H2	3	0	0.0-0.20	0.10
548 O111 : K58 : H2	8	0	0.0-0.16	0.02
8910 O111 : K58 : H2	8	0	0.0-0.23	0.03
1062 O111 : K58 : H-	4	0	0.0-0.0	0.0
1065 O111 : K58 : H-	7	1	0.0-1.25	0.26
1066 O111 : K58 : H-	6	2	0.0-0.83	0.29
12 O111 : K58 : H.	7	2	0.0-1.00	0.31
56899 O114 : K90 : H-	9	0	0.0-0.20	0.06
438 O4 : - : H5	3	0	0.0	0.0
439 O4 : - : H5	3	0	0.0	0.0
466 O4 : - : H5	4	0	0.0	0.0
Kr O4 : - : H5	8	3	0.0-1.00	0.27
<i>2. Supernatants (Sd):</i>				
3 O26 : K60 : H11	21	20	0.36-1.50	0.94
4 O26 : K60 : H11	18	17	0.17-2.00	0.96
12 O111 : K58 : H?	5	0	0.0-0.38	0.25
E-5 O78 : K80 : H-	10	0	0.0-0.18	0.04
56899 O114 : K90 : H-	10	0	0.0-0.18	0.08
1065 O111 : K58 : H-	2	0	0.05-0.18	0.12
1066 O111 : K58 : H-	4	1	0.0-1.50	0.51
Kr O4 : - : H5	4	0	0.0-0.33	0.14
<i>3. Whole cell lysates (WCL):</i>				
3 O26 : K60 : H11	5	3	0.31-0.91	0.58
4 O26 : K60 : H11	9	7	0.18-1.60	1.02
1066 O111 : K58 : H-	3	0	0.0	0.0
12 O111 : K58 : H?	5	0	0.0-0.31	0.14

loops was controlled and compared to the germ count of the initial inoculum. Results are summarized in Table IV. It could be stated that no substantial change occurred in the bacterial count of the test strains. In contrast, dividing bacteria could be seen in the light and electron micrographs.

Table IV

Average and range of germ count in inocula and fluid contents of dilated loops

Strain	Serotype	Challenge dose	Germ count in content of dilated loop	
			9 hr postchallenge	18 hr postchallenge
B7A	O148 : H28	1.33×10^{10} ($0.73-2.0 \times 10^{10}$)	1.42×10^{10} ($0.26-8.6 \times 10^{10}$)	1.45×10^{10} ($1.0-1.9 \times 10^{10}$)
3	O26 : K60 : H11	1.16×10^{10} ($0.5-2.2 \times 10^{10}$)	0.88×10^{10} ($0.65-1.3 \times 10^{10}$)	2.5×10^{10} —
4	O26 : K60 : H11	0.43×10^{10} ($0.40-0.46 \times 10^{10}$)	1.13×10^{10} ($0.82-1.6 \times 10^{10}$)	—

Light microscopy revealed loop-negative EEC-I strains sometimes attached to the brush border of the unaffected epithelial lining (Fig. 1a, b). However, in most cases the organisms were not only attached to the brush border surface of the enterocytes, but also were seen inside the brush border and in the cytoplasm of absorptive and goblet cells (Fig. 2). Single bacteria were discovered also in the lamina propria. Mild focal leukocyte infiltration of the villi occurred but congestion and oedema were insignificant in these areas.

Gut-loop-positive EEC-I strains Nos 3 and 4 (O26 : K60 : H11) often adhered to the epithelial cells, multiplied on their surface and penetrated into them (Fig. 3a, b, c). When a great number of bacteria adhered to the epithelial cells they sometimes resulted in disjoining and shedding of enterocytes into

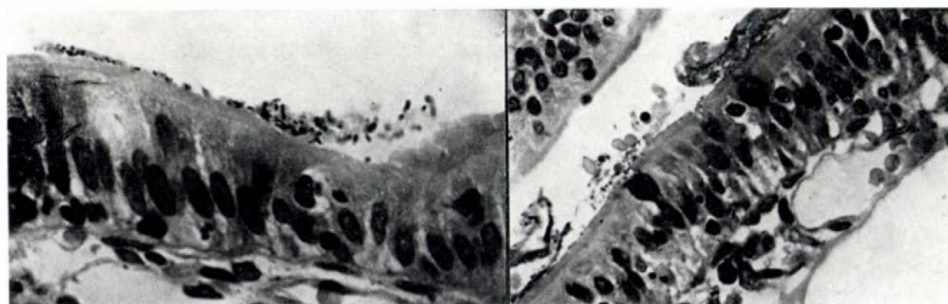


Fig. 1. Attachment to the brush border surface of organisms of loop-negative strains. (a) Strain E-5 (O78 : K80 : H-); 9 hr postchallenge, eosin-azure, $\times 850$; (b) Strain 1066 (O111 : K58 : H-, occasionally loop-positive); 10 hr postchallenge, eosin-azure, $\times 800$



Fig. 2. Organisms of loop-negative EEC-I strain 558 (O111 :K58 :H2) attached to the brush border penetrate across it and the apical cytoplasm; 9hr postchallenge, 1 μ m Araldite section, toluidine blue and azure. $\times 1500$

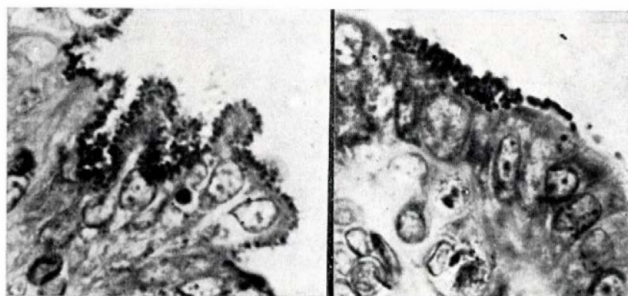
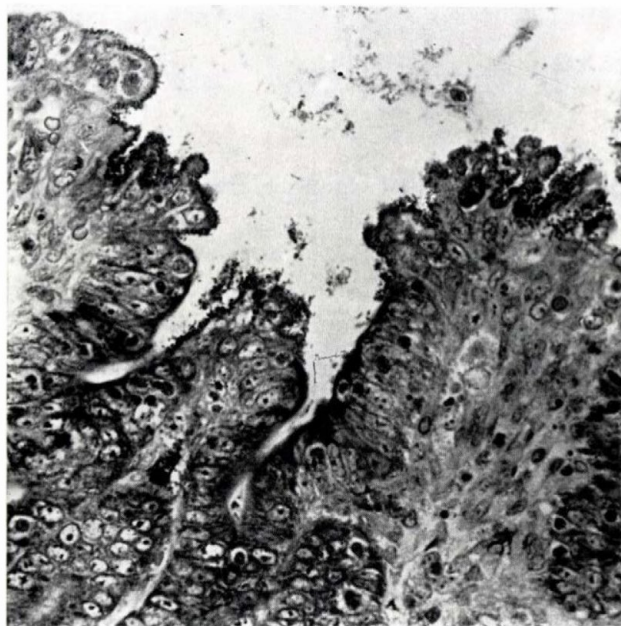


Fig. 3. Organisms of enterotoxigenic EEC-I strain 3 (O26; K60 :H11) densely cover the brush border, intruding into it and disjoining some shedding enterocytes and penetrate into the epithelial cells and lamina propria; 18 hr postchallenge, eosin-azure. (a) $\times 690$; (b) magnified from upper left of (a) $\times 1200$; (c) $\times 1200$

the lumen. Mild focal polymorphonuclear infiltration was noted in midvillous core. Oedema and congestion of the lamina propria were pronounced and the picture was similar to the results obtained with ETEC, although some EEC-I organisms and leukocytes were observed in the oedematous tissue of the lamina propria, which was never found in the case of ETEC strains except in necrotic mucosal areas due to hydrostatic pressure after 18–24 hr.

Electron microscopic examinations showed that the organisms of loop-negative EEC-I strains were attached to the epithelial cell surface among the microvilli in close contact with outer cell membrane (Fig. 4a). Some bacteria indented the cell membrane forming invaginations, and single organisms were found to have penetrated into the cytoplasm, being enclosed in membrane-bound phagosome-like vacuoles (Fig. 4b).

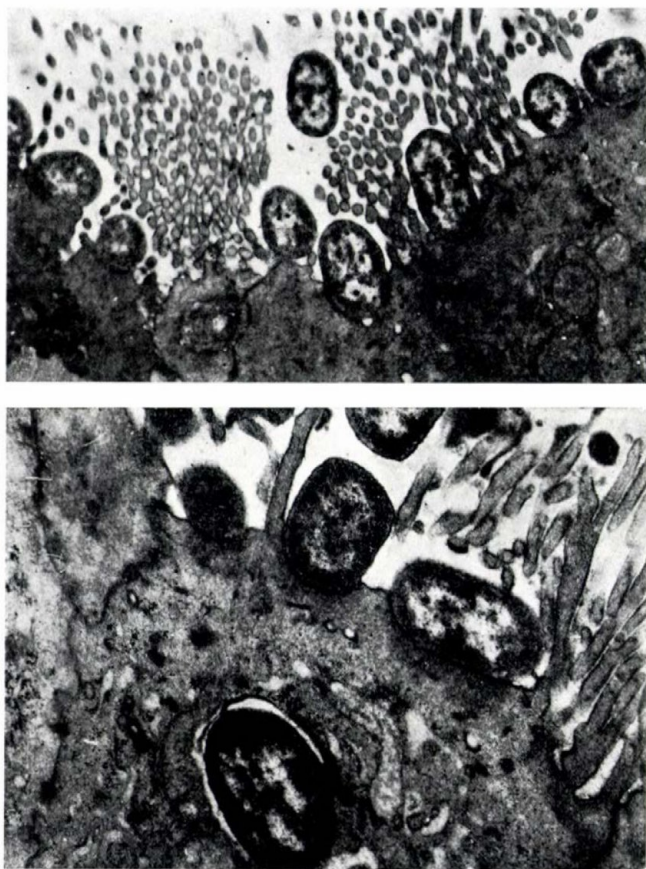


Fig. 4. Organisms of loop-negative EEC-I strain 548 (O111 : K58 : H2) (see Fig. 2 light micrograph) intrude between the microvilli, multiply on the outer enterocyte membrane making close contact with and indenting it at sites; one organisms (b) has penetrated into the cytoplasm being enclosed in the membrane-bound phagosome-like vacuole; 9 hr postchallenge. (a) $\times 12\ 000$; (b) $\times 24\ 000$

Electron microscopic findings were more marked in the case of loop-positive EEC-I strains Nos 3 and 4 (O26 : K60 : H11). Adhering to and multiplying on the outer enterocyte membrane these bacteria caused a disappearance of the microvilli. They penetrated into the cytoplasm more often and in greater amounts, being enclosed in membrane-bound phagosome-like vacuoles singly or in clusters (Fig. 5a, b). Some of these phagosomes contained lysosomes and cellular organelles (Fig. 5a), while others contained only the bacteria. Around the latter phagosomes the mitochondria were often in close contact with the phagosome membrane (Fig. 5b). The bacteria displayed no changes.

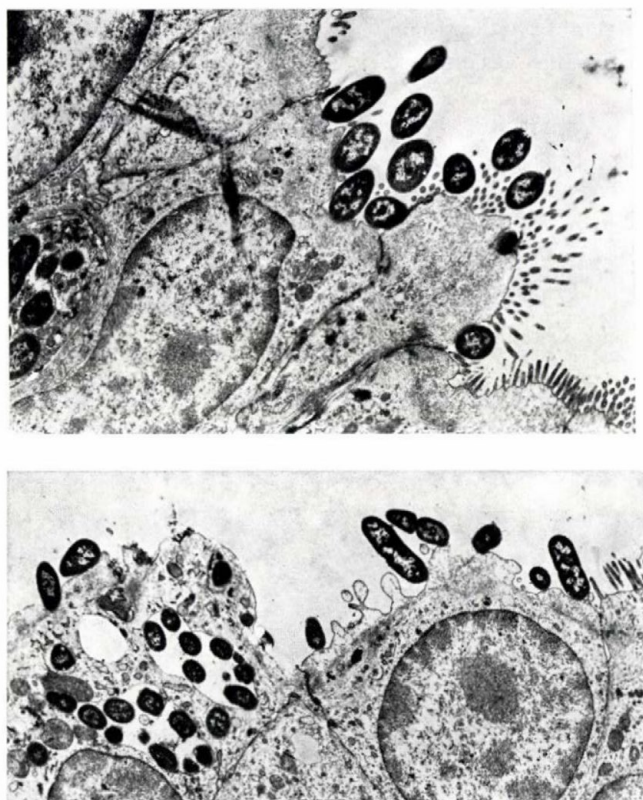


Fig. 5. Numerous organisms of enterotoxigenic EEC-I strain 3 (O26 : K60 : H11) multiply on denuded outer enterocyte membrane. They are attached to it and indent it. Part of the bacteria have penetrated into the cytoplasm, being enclosed, singly or in clusters, in membrane-bound phagosome-like vacuoles; 18 hr postchallenge. (a) $\times 7000$; (b) $\times 5700$. Note different appearance of phagosomes in (a) and (b) and the disappearance of the terminal web, with formation of apical cytoplasm projections

Alterations of the enterocyte apical cytoplasm and of the cellular secretory apparatus were observed regularly. The terminal web was often greatly enlarged or blurred, the apical cytoplasm was rarified and formed projections

or bleb-like bulgings (Fig. 5a, b; Fig. 6a, b). The endoplasmic reticulum and Golgi apparatus were frequently hypertrophic and dilated, particularly in low-differentiated enterocytes and undifferentiated crypt cells, which contained an increased number of secretory granules to be discharged into the intestinal lumen. The same manifestations of increased enterocyte secretion were observed in the experiments with S and WCL preparations of the two EEC-I strains at issue and also with ETEC living cultures (Fig. 7) and their enterotoxins (Fig. 8).

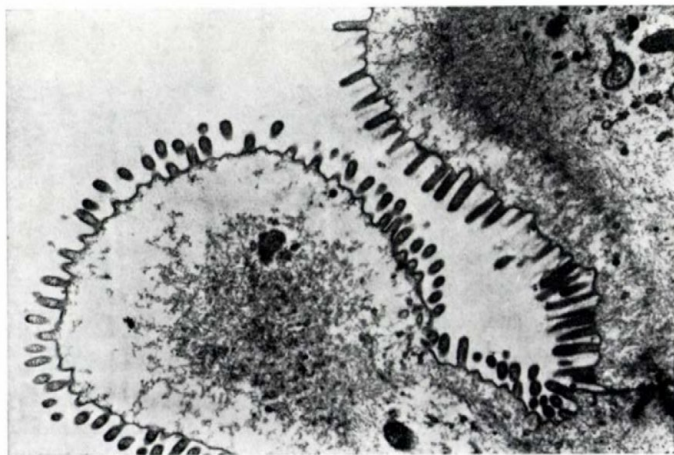


Fig. 6. Enterocyte apocrine secretion of bleb-like cytoplasmic projections 18 hr postchallenge with enterotoxigenic EEC-I strain 3 (O26 : K60 : H16). (a) $\times 10\ 000$; (b) $\times 14\ 000$

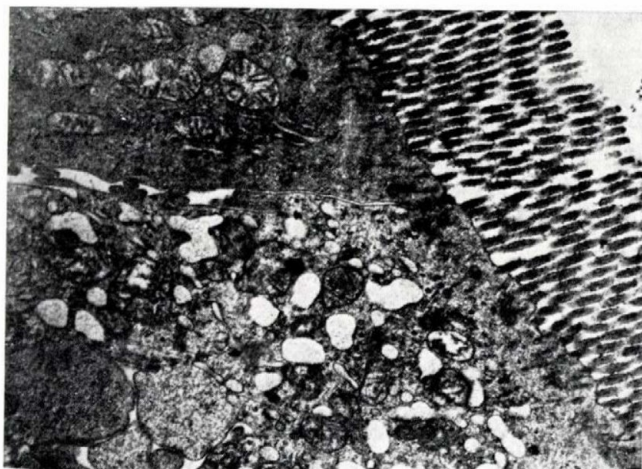


Fig. 7. Somewhat enlarged terminal web, greatly distended hypertrophic endoplasmic reticulum and Golgi apparatus, numerous hypertrophic mitochondria in the enterocytic cytoplasm; 6 hr postchallenge with ETEC strain B2C (O6 : H16). $\times 15\,000$

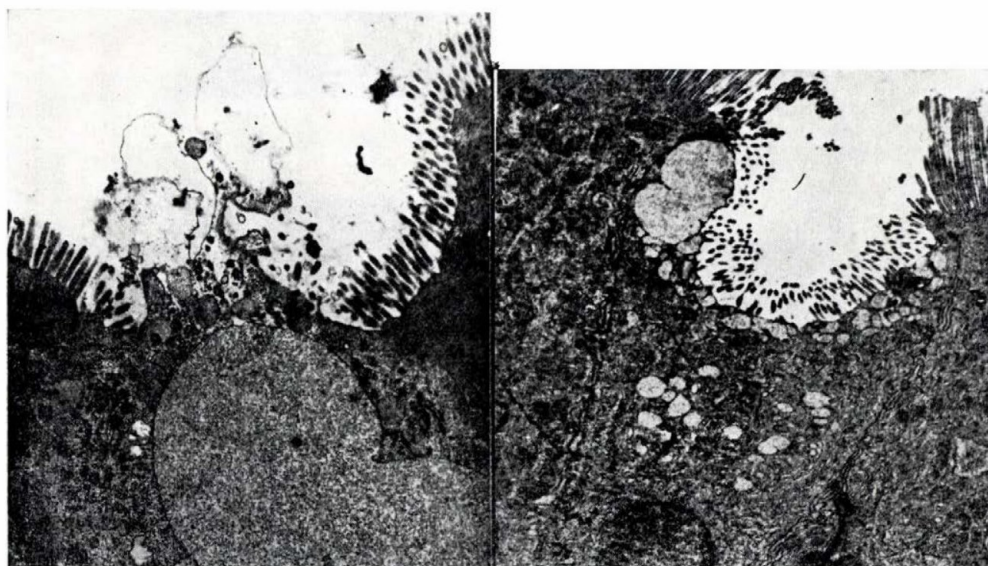


Fig. 8. Enhanced merocrine and apocrine secretion of epithelial cells 18 hr postexposure to WEL enterotoxin of ETEC strain B7A (O148 : H28). (a) Large fused secretory granules in low-differentiated enterocyte in the centre and at right bottom, the overlying apical cytoplasm forms bleb-like projections discharging apocrine secretion into the lumen; $\times 7000$. (b) Small and fused large secretory granules in the undifferentiated crypt cell are discharging merocrine secretion into the lumen; $\times 8500$.

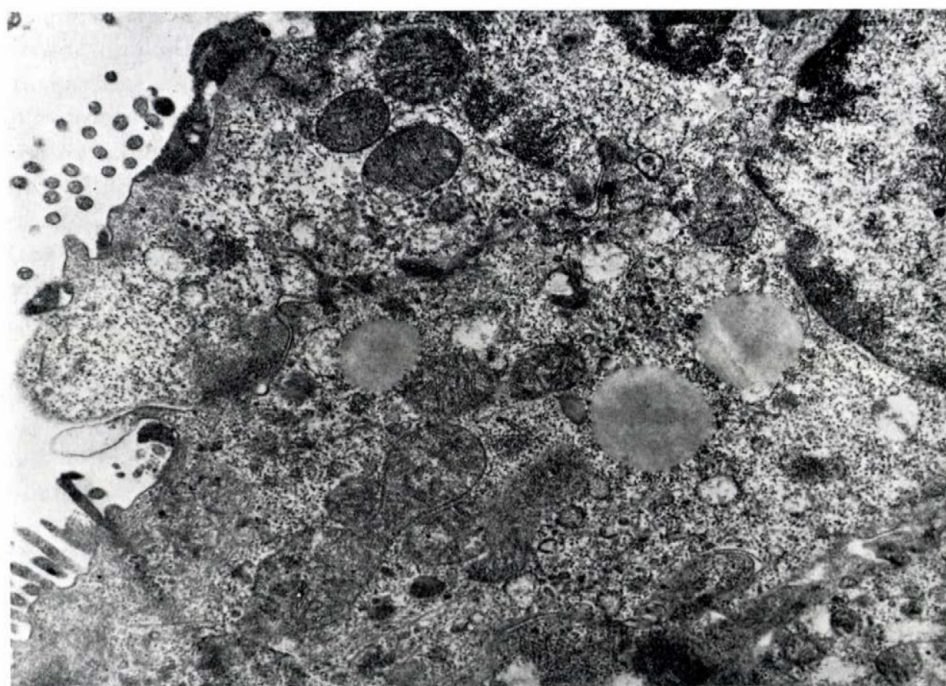


Fig. 8. (c) Apical cytoplasm of two adjacent enterocytes forms projections to be discharged by apocrine secretion; $\times 10\,000$

Discussion

DE *et al.* [22] reported that *E. coli* strains isolated from adult patients with typical cholera syndrome could induce mucosal necrosis with fluid accumulation and dilatation of the ligated rabbit intestinal loop. Three EEC-I strains from infantile diarrhoea (O111, O55, O26) induced the same picture. Using this model, TAYLOR *et al.* [18] showed that EEC-I strains adhered to the epithelial lining and induced an inflammatory reaction depending on their virulence. These authors failed to observe mucosal penetration but noted some necrotic changes in dilated loops. Human and experimental cholera studies of FRESH *et al.* [23] revealed that the necrosis, considered earlier characteristic of cholera, was a *post mortem* change and due to hydrostatic pressure of the mucosa in the ligated rabbit intestinal loop. DRUCKER *et al.* [24] observed not only attachment of EEC-I strains to the intestinal epithelium and mucosal inflammation, but also their penetration through the epithelial lining into the lamina propria and the blood. Consistent changes were observed by ILGNER [25] in infants who died of *E. coli* enteritis. STALEY *et al.* [26] demonstrated

by electron microscopy how after oral infection of germ-free newborn piglets the EEC-I organisms penetrated through the intestinal epithelium. SMITH and HALLS [27] and GYLES and BARNUM [28] showed that causative agents of animal colibacillosis elaborate heat-stable and heat-labile enterotoxins in the ligated intestinal loops of different animal species. Some similar enterotoxin-like substances were detected in certain EEC-I strains isolated from human infants by SMITH and GYLES [13]. It is emphasized that the capacity of EEC-I to penetrate through the epithelial cells cannot be compared with penetration and intra-epithelial multiplication of shigella-like EEC-II, the causative agents of dysentery-like diseases in adults and children [3, 7, 29-32].

Current studies of human ETEC were initiated by DUPONT *et al.* [3], GORBACH *et al.* [11] and SACK *et al.* [12] who stressed the similarity in the mode of pathogenic action between enterotoxins of ETEC and that of cholera vibrios. DUPONT *et al.* [3] have, however, shown that human ETEC isolated from adult patients do not penetrate the intestinal epithelial lining and do not cause inflammation or any conspicuous lesions. The properties of different EEC and the action of these enteropathogens and the intestinal epithelium is still unclear [5, 6].

Some authors deny the invasive potential of EEC-I and even that of salmonellae and term the EEC-I group salmonella-like EEC group [2, 30] or call these bacteria together with the ETEC group non-invasive EEC, in contrast to invasive shigella-like EEC [33, 34]. Others believe EEC-I to be invasive with a potential similar to that of salmonellae [35]. They also consider ETEC as well as cholera vibrios to be incapable of penetrating the intestinal mucosa or to attach to the epithelium [35]. Nonetheless, the importance of the capacity of cholera vibrios and ETEC, pathogenic for both man and animals, to attach to the epithelial brush border has been proved without doubt [4-10, 36, 37-39].

Our previous electron microscopic studies have shown that ETEC organisms in the intestinal loops behaved like cholera vibrios [7-10]. They attach first to the glycocalyx layer, covering the microvilli of the enterocytes, multiply on the brush border surface without causing an inflammatory reaction except for the initial appearance of a few polymorpho-nuclears. Congestion, oedema and fluid accumulation with loop dilatation result not from inflammation as is the case with penetrating shigellae, salmonellae and shigella-like EEC-II O124 [6, 7], but from epithelial hypersecretion, that appears identically in loops challenged with *Vibrio cholerae* var. *el-tor* [9] and exposed to ETEC living cultures, S and WCL enterotoxins [10]. The same was observed in the present study. The manifestations of enhanced mucrocrine secretion of the epithelium were mucus depletion of most goblet cells and discharge of an increased number of secretory granules of low-differentiated enterocytes

and undifferentiated crypt cells. Some similar findings in experiments with cholera toxin and enterotoxins of porcine ETEC in ligated rabbit and swine intestinal loops were reported by MOON *et al.* [40]. The manifestations of enhanced apocrine secretion in our experiments were frequent bleb-like apical cytoplasmic projections of some enterocytes preceded by terminal web enlargement and disappearance. These findings resemble the pattern seen in small intestinal specimens of cholera patients [41, 42] and even in the healthy human gut during pilocarpine-induced secretion [43].

Contrary to ETEC strains, common EEC-I organisms in the present study did not cause any sign of epithelial hypersecretion nor loop dilatation, as was the case in our previous experiments [6, 7]. Multiplying on the brush border they intruded between the microvilli and caused their shedding, made close contact with the outer cell membrane, and penetrated deep between the epithelial cells and the lamina propria inducing mild focal inflammation. This situation agreed with former light microscopic findings in rabbit intestinal loops [24], in infants died of *E. coli* enteritis [25] and with electron microscopic findings in orally challenged piglets [26].

We have found two enterotoxigenic EEC-I strains of the O26 : K60 : H11 serotype. Like common EEC-I strains, these bacteria adhered to the epithelial cells, multiplied on the enterocyte outer membrane surface and penetrated into the cytoplasm becoming enclosed in membrane-bound vacuoles. They reached the lamina propria, *i.e.* behaved to some extent like salmonellae in orally challenged guinea pigs as observed by TAKEUCHI [35] and also in our previous studies [7]. At the same time these EEC-I organisms and their enterotoxins caused pronounced epithelial hypersecretion.

Little is known of the effect of enterotoxigenic EEC-I strains in infants. GORBACH and KHURANA [44] and KANTOR *et al.* [45] observed that some, mostly untypable, *E. coli* strains isolated from sick infants elaborated less enterotoxin after oral infection of infantile rabbits than in the intestinal loops of adult animals. EVANS *et al.* [46] using PF activity intradermal assay found weaker and unusual toxin production by those strains, even when concentrated preparations were used. SACK *et al.* [47] have been unable to find strains which were positive in the infantile rabbit but not in the other assays including the adult-rabbit-loop test and the more sensitive Y1 adrenal-cell assay. Neither could ZINKERNAGEL and COLOMBINI [48] find any enterotoxic activity of S and WCL preparations of the EEC-I strains that induced accumulation of 2–6 ml/10 cm of fluid in the ligated intestinal loop. Special surveys of EEC-I strains which caused infantile diarrhoea in England and U.S.A. have verified the role of *E. coli* [47, 49, 50]. Occasionally, EEC-I serotypes capable of elaborating enterotoxins were isolated from adults and children with cholera-like disease [11, 12, 51, 52]. These data, however, have not been supported by morphological studies.

The data presented indicate that strains capable of producing enterotoxins occur but are infrequent among EEC-I, the pathogens of infantile diarrhoea. According to our morphologic studies, the main difference between enterotoxin-elaborating EEC-I strains and the ETEC the enterotoxigenic pathogens for adults and children rather than for infants, is the invasive property of the former group. In current literature, however, the term invasiveness is sometimes used equivocally, particularly if the property is not supported by morphologic studies *in vivo*. RUDOX and NELSON [53] tested in HEP-2 cell monolayers untypable *E. coli* strains isolated from healthy infants and children, from infants with diarrhoea and observed the penetration into cultivated cells by the agents isolated from 30% sick and 12% healthy subjects. Whether the strains were really invasive *in vivo*, and in particular, whether they were pathogens of the disease, remained, however, unclear. These authors tested also the enterotoxigenic capacity of the strains using the suckling-mice assay developed by DEAN *et al.* [19] who could not find any enterotoxigenic *E. coli* in infants and children with diarrhoea. RUDOX and NELSON [53] detected such enterotoxigenic untypable *E. coli* strains in 86% diarrhoeic and 41% healthy infants and children by the said test, but the values for the ratio bowel per body weight regarded by these workers as positive were as low as from 0.071 to 0.078.

NETER [54] summarized that despite the great progress in the study of enteropathogenicity of *E. coli*, many aspects should be investigated for the better understanding of its pathogenicity, particularly in infants and children. Among others, elucidation of the clinical significance of the enterotoxigenicity of certain strains of EEC-I must await further studies.

Acknowledgements. We are indebted to Dr. K. B. GRABOVSKAYA, and Dr. L. E. RAVDONIKAYA (Department of Microbiology and Immunology, Institute of Experimental Medicine, U.S.S.R. Academy of Medical Sciences, Leningrad) for help in preparing and conducting some experiments; to Dr. P. GECK (National Institute of Public Health, Budapest), Dr. B. RÉDEY (Public Health Station, Veszprém), Dr. H. L. DUPONT (Division of Infectious Diseases, University of Maryland, Baltimore, Maryland, U.S.A.), Dr. S. L. GORBACH (Infectious Diseases Section, Veterans Administration Hospital, Sepulveda, California, U.S.A.), Dr. H. W. SMITH (Animal Health Trust, Stock, Essex, England) for kind supply of strains.

REFERENCES

1. NOVGORODSKAYA, E. M., AYDEEVA, T. A., ARBUZOVA, V. A.: Abstr. 9th Int. Congr. Microbiology, Moscow 1966, p. 718.
2. SAKAZAKI, R., TAMURA, K., SAITO, M.: Jap. J. med. Sci. **20**, 387 (1967).
3. DUPONT, H. L., FORMAL, S. B., HORNICK, R. B., SNYDER, M. J., LIBONATI, J. P., SHEAHAN, D. G., LABREC, E. H., KALAS, J. P.: New Engl. J. Med. **285**, 1 (1971).
4. MOON, H. W.: Advanc. vet. Sci. **18**, 179 (1974).
5. NOVGORODSKAYA, E. M., POLOTSKY, YU. E.: In VOINO-YASENETSKY, M. V., BAKÁCS, T. (eds): Pathogenesis of Intestinal Infections. Akadémiai Kiadó, Budapest. 1977. p. 257.
6. POLOTSKY, YU. E.: In VOINO-YASENETSKY, M. V., BAKÁCS, T. (eds): Pathogenesis of Intestinal Infections. Akadémiai Kiadó, Budapest. 1977. p. 296.
7. ПОЛОЦКИЙ, Ю. Е., ДРАГУНСКАЯ, Е. М., СНИГИРЕВСКАЯ, Е. С., ВОЙНО-ЯСЕНЕЦКИЙ, М. В.: Вестн. Акад. Мед. Наук СССР **1**, 8 (1976).

8. ПОЛОЦКИЙ, Ю. Е., СНИГИРЕВСКАЯ, Е. С., ДРАГУНСКАЯ, Е. М.: Бюлл. exper. биол. 6 110 (1974).
9. POLOTSKY, Yu. E., DRAGUNSKAYA, E. M., SAMOSTRELSKY, A. Y., VASSER, N. R., EFREMOV, V. E., SNIGIREVSKAYA, E. S., SELIVERSTOVA, V. G.: Med. Biol. (Helsinki). 55, 130. (1977).
10. ПОЛОЦКИЙ, Ю. Е., ДРАГУНСКАЯ, Е. М., СНИГИРЕВСКАЯ, Е. С., СЕЛИВЕРС-ТОВА, В. Г.: Арх. Пат. (Москва), 2, 10 (1977).
11. GORBACH, S. L., BANWELL, J. G., CHATTERJEE, B. D., JACOBS, B., SACK, R. B.: J. clin. Invest. 50, 881 (1971).
12. SACK, R. B., GORBACH, S. L., BANWELL, J. G., JACOBS, B., CHATTERJEE, B. D.: J. infect. Dis. 123, 378 (1971).
13. SMITH, H. W., GYLES, C. L.: J. med. Microbiol. 3, 403 (1970).
14. CZIRÓK, É., MILCH, H., MADÁR, J., DOMBI, I., ADAMIS, É.: Egészségtudomány 19, 85 (1975).
15. CZIRÓK, É., MILCH, H., STVERTECZKY, Z., HÉRMÁN, G., LOSONCZY, G.: Acta microbiol. Acad. Sci. hung. 22, 299 (1975).
16. SAKAZAKI, R., TAMURA, K., NAKAMURA, A., KURATA, T.: Jap. J. med. Sci. 27, 45 (1974).
17. АВДЕЕВА, Т. А., ПОЛОЦКИЙ, Ю. Е., СМИРНОВА, Л. А., ДРАГУНСКАЯ, Е. М., ПОЯСОВА, Е. В., ЧАЛЕНКО, В. Г.: Ж. микробиол. (Москва). 11, 9 (1973).
18. TAYLOR, J., MALTBY, M. P., PAYNE, J. M.: Path. Bact. 76, 491 (1958).
19. DEAN, A. G., CHING, Y. C., WILLIAMS, R. G., HARDEN, L. B.: J. infect. Dis. 125, 407 (1972).
20. JACKS, T. M., WU, B. J.: Infect. Immun. 9, 342 (1974).
21. EVANS, D. G., EVANS, D. J. JR., GORBACH, S. L.: Infect. Immun. 8, 725 (1973).
22. DE, S. N., BHATTACHARYA, D., SARKAR, J.: J. Path. Bact. 71, 201 (1956).
23. FRESH, J. W., VERSAGE, P. M., REYES, V.: Arch. Path. 77, 529 (1964).
24. DRUCKER, M. M., YEIVIN, R., SACKS, T. G.: Israel J. med. Sci. 3, 445 (1967).
25. ILGNER, G.: In ADAM, A. (ed.): Säuglings-Enteritis. G. Thieme, Stuttgart 1956. p. 172.
26. STALEY, T. E., JONES, E. W., CORLEY, L. D.: Amer. J. Path. 56, 371 (1969).
27. SMITH, H. W., HALLS, S.: J. Path. Bact. 93, 531 (1967).
28. GYLES, C. L., BARNUM, D. A.: J. infect. Dis. 120, 419 (1969).
29. ПОЛОЦКИЙ, Ю. Е., АРБУЗОВА, В. А.: Ж. микробиол. 1, 61 (1967).
30. OGAWA, H., NAKAMURA, A., SAKAZAKI, R.: Jap. J. med. Sci. 21, 333 (1968).
31. ПОЛОЦКИЙ, Ю. Е., ВАСЦЕР, Н. Р.: Бюлл. эксп. биол. 2, 20 (1970).
32. ПОЛОЦКИЙ, Ю. Е., ВАСЦЕР, Н. Р., ДРАГУНСКАЯ, Е. М.: Ж. микробиол. (Москва) 12, 76 (1971).
33. SAKAZAKI, R., TAMURA, K., NAKAMURA, A.: Jap. J. med. Sci. 27, 7 (1974).
34. SAKAZAKI, R., TAMURA, K., NAKAMURA, A., KURATA, T., GHODA, A., TAKEUCHI, S.: Jap. J. med. Sci. 27, 19 (1974).
35. TAKEUCHI, A.: In SCHLESSINGER, D. (ed.): Microbiology. American Society of Microbiology, Washington, D. C. 1975. p. 174.
36. FRETER, R.: Texas Rep. Biol. Med. 27, Suppl. 1, 299 (1969).
37. HOHMANN, A., WILSON, M. R.: Infect. Immun. 12, 866 (1975).
38. SCHRANK, G. D., VERWEY, W. F.: Infect. Immun. 13, 195 (1976).
39. EVANS, D. G., SILVER, R. P., EVANS, D. J. JR., CHASE, D. G., GORBACH, S. L.: Infect. Immun. 12, 656 (1975).
40. MOON, H. W., WHIPP, S. C., BAETZ, A. L.: Lab. Invest. 25, 133 (1971).
41. CHEN, H. C., REYES, V., FRESH, J. W.: Virchows Arch. ges. Physiol. Abt. A. Zellpath. 7, 236 (1971).
42. ASAKURA, H., TSUCHIYA, N., WATANABE, Y., ENOMOTO, Y., MORITA, A., MORISHITA, T., FUKUMI, H., OHASHI, M., CASTRO, A., UYLANGCO, C.: Gut 15, 531 (1974).
43. TRIER, J. S., RUBIN, C. E.: Gastroenterology 49, 574 (1965).
44. GORBACH, S. L., KHURANA, C. M.: New Engl. J. Med. 287, 791 (1972).
45. KANTOR, H. S., TAO, P., GORBACH, S. L.: J. infect. Dis. 129, 1 (1974).
46. EVANS, D. G., EVANS, D. J. JR., GORBACH, S. L.: Infect. Immun. 8, 731 (1973).
47. SACK, R. B., HIRSCHHORN, N., BROWNLEE, I., CASH, R. A., WOODWARD, W. E., SACK, D. A.: New Engl. J. Med. 292, 1041 (1975).
48. ZINKERNAGEL, R. M., COLOMBINI, A.: Med. Microbiol. Immunol. 162, 1 (1975).
49. GROSS, R. J., SCOTLAND, S. M., ROWE, B.: Lancet 1, 629 (1976).
50. ROWE, B., GROSS, R. J., SCOTLAND, S. M.: Lancet 2, 37 (1976).
51. ØRSKOV, F., ØRSKOV, I., EVANS, D. J. JR., SACK, R. B., SACK, D. A., WADSTRÖM, T.: Med. Microbiol. Immunol. 162, 73 (1976).
52. MORRIS, G. K., MERSON, M. H., SACK, D. A., WELLS, J. G., MARTIN, W. T., DEWITT, W. E., FEELEY, J. C., SACK, R. B., BESSUDO, D. M.: J. clin. Microbiol. 3, 486 (1976).

53. RUDOY, R. C., NELSON, J. D.: Amer. J. Dis. Child. **129**, 668 (1975).
54. NETER, E.: Amer. J. Dis. Child. **129**, 666 (1975).

Address of the authors:

Yu. E. POLOTSKY, E. M. DRAGUNSKAYA, V. G. SELIVERSTOVA
Department of Pathological Anatomy, Institute of Experimental Medicine, U.S.S.R. Academy of Medical Sciences
Kirovsky prospekt 69/71 Leningrad P-22, 197022 U.S.S.R.

T. A. AVDEEVA, M. G. CHAKHUTINSKAYA, N. V. SAFONOVA, E. I. KARYAGINA
Department of Enteric Infections, Pasteur Institute of Epidemiology and Microbiology
ul. Mira 14, Leningrad P-101, 197101 U.S.S.R.

IVÁN KÉTYI, ADELE VERTÉNYI, BÉLA RALOVICH, LEVENTE EMŐDY, ILONA MÁLOVICS
Institute of Microbiology, University Medical School
Szigeti út 12, H-7643 Pécs, Hungary

E. S. SNIGIREVSKAYA
Membrane Ultrastructure Group, Institute of Cytology, U.S.S.R. Academy of Sciences
Prospekt Maklina 22, Leningrad, U.S.S.R.

CHANGES IN THE SURFACE LAYER (SHEATH) OF THE CELL WALL OF STREPTOMYCETES DURING SPORULATION

I. M. SZABÓ, L. KONDICS, MÁRIA MARTON and ILONA BUTI

Department of Microbiology, Eötvös L. University, Budapest, Hungary

(Received November 2, 1976)

Fine structural changes of the sheath, occurring during spore formation in strains *Streptomyces finlayi* ATCC 23 340 and *Streptomyces coeruleorubidus* FBUA 328 were investigated by means of electron microscopy of air-dried whole mounts and thin sections. The results suggest that in the strains the process of sporulation is not strictly synchronized spatially with the molecular arrangements and re-arrangements (formation of hairy or spiny surface ornaments) occurring outside the wall of the sporulating hyphae, in the sheath. On the contrary, the intensity of transformation induction in the sheath may show a gradually decreasing tendency from the tip of the sporulating hypha towards its sterile basal part, resulting in the formation of both ornamented and smooth spores in the same chain. This suggests that the morphogenetic changes occurring in the sheath during spore formation are probably controlled by a "functional centre", located near the tip of the sporulating hypha, and this centre is perhaps identical with the cell unit at the tip.

In 1954, FLAIG *et al.* [1] demonstrated that some *Streptomyces* have spores with warty, spiny or hairy surfaces. These ornaments develop from the sheath as spore delimitation occurs [2, 3]. RANCOURT and LECHEVALIER [4] studying the mode of formation of conidia by a strain of *Streptomyces viridochromogenes* found that the laminated hyphal wall is covered by a fibrous non-spiny layer. During the process of sporulation this outer fibrous sheath is transformed drastically by the elaboration of spines and, in addition, it breaks up into spore size segments that covers each spore as a mantle. Consequently, in contrast to the spineless sheath of non-sporulating hyphae, marked changes occur in the surface layer of the sporulating hyphae of *S. viridochromogenes* (molecular processes involved in the transition from smooth to spiny sheath). According to WILDERMUTH [5] unsegmented hyphae bearing spines or spine rudiments represent hyphae undergoing sporulation. These morphogenetic changes are raising fascinating problems concerning the processes responsible for such molecular arrangements occurring outside the wall of the hyphae [5].

The striking structural difference between the surface "membranes" of non-sporulating aerial hypae and of the spores which were found by WILDERMUTH [6] in a number of non-smooth spored streptomycetes seems to be a general feature of these organisms.

Spiny or hairy ornamentations on the spore surface and smooth spores without surface ornamentation (as revealed by electron microscopy) have

generally been regarded as stable and readily distinguishable characteristics. Therefore, spore silhouettes are used as a standard criterion for the morphological characterization of species [7] and new isolates. Nevertheless, occasionally a few smooth spores occurred in the spore chains of some non-smooth spored streptomyces. According to the evaluation of criteria used in the ISP cooperative description of *Streptomyces* and *Streptoverticillium* type strains [8], from among 436 type, neotype or reference strains, only 10 (2.3%) had also produced smooth spores among the characteristic spiny ones, and smooth spores among the hairy ornamented conidia were observed solely in the type culture of *S. finlayi*. Unfortunately, little information is available in the literature on the formation, morphogenesis and frequency of occurrence of smooth spores in the sporulating aerial mycelium of non-smooth spored streptomyces. The thin sheath, which is responsible for the ornamentation of spores, does not always persist and coat each detached spore but, as e.g. in *S. venezuelae* and *S. griseus*, the spores mature within a loose sheath and become detached from the chain free from sheath material [9]. According to WILLIAMS *et al.* [10], the hairy sheath is usually coating the detached spores in *S. finlayi*, but occasionally release of spores after the partial disruption of the hairy sheath results in the occurrence of both hairy and smooth spores in the aerial mass. On studying a freshly isolated strain of *S. finlayi*, SZABÓ *et al.* [11] were able to confirm the above findings of WILLIAMS *et al.* [10].

The aim of the present study was to obtain more information about the morphogenetic changes occurring in the sheath of hyphae during sporulation. Evidence has been presented that in *S. coeruleorubidus* and *S. finlayi* the changes in the fine structure of the sheath may show a particular pattern of morphological events suggesting the existence of a possible regulatory mechanism operating over a certain length of the sporulating hypha and having a centre near to its tip.

Materials and methods

Bacterial strains. Strain FBUA (Forschungsinstitut für Bodenkunde der Ungarischen Akademie der Wissenschaften, Budapest) 328 of *S. coeruleorubidus* was isolated by A. N. IBRAHIM (Cairo, Egypt) from a chernozem soil in Hungary. The procedures employed for the study of the cultural and morphological properties and carbon utilization abilities of strain 328 were the same as proposed in the International Streptomyces Project [7]. The range of growth temperature was determined on oatmeal agar slants incubated at 3, 6, 28, 37, 39, 41 and 42°C. Salt tolerance was tested in glycerol-glucose-asparagine broth with 2, 4, 6, 7, 8, 9 and 10% NaCl. Sensitivity to antibiotics was assayed by sensitivity discs (Institute for Serobacteriological Production and Research, Human, Budapest).

Strain 328 produces well developed spirals (Section Spirales). Mature spore chains contain 10 to 50 or more spores per chain. Aerial mass colour is in the Gray colour series on yeast-malt agar and in the Blue colour series on oatmeal agar. The reverse side of colony is yellow-brown or yellow-brown + red. This pigment is not a pH indicator. Colour in medium: melanoid pigments are formed in peptone-yeast-iron agar and tyrosine agar. Red pigment, not sensitive to pH changes, is found in the medium with cultures grown in oatmeal agar and glycerol-asparagine agar. Carbon utilization: L-arabinose, rhamnose, D-xylose, D-glucose,

D-fructose, lactose, melibiose, trehalose, sucrose, raffinose, inulin, adonitol, i-inositol, D-mannitol, D-sorbitol and salicin are utilized for growth. No growth or trace of growth on dulcitol and melizitose. Cellulose and paraffin not utilized. No growth with syringaldehyde and only trace of growth with vanillin. Nitrates reduced in nitrate broth. Starch is hydrolysed. Proteolytic activity: positive. Tween 80 is split. Salt tolerance: good growth with 0 to 7% NaCl; no growth in 9% NaCl. Temperature range of growth: minimum 3–5°C; maximum: 38–39°C. Good growth between 6 and 37°C. Antagonistic properties: Ineffective against *Aspergillus niger*, *Saccharomyces carlsbergensis*, *Escherichia coli* and *Bacillus subtilis*. Sensitivity to antibiotics: Sensitive to oxytetracycline (30 µg), chlortetracycline (30 µg), spiramycin (30 µg), streptomycin (30 µg), novobiocin (30 µg), neomycin (100 µg), kanamycin (30 µg), vancomycin (50 µg), chloramphenicol (30 µg), tetracycline (30 µg). Resistant to oxacillin (10 µg), erythromycin (10 µg) oleandomycin (30 µg), penicillin (3 IU), methicillin (20 µg) and polymyxin-B (15 µg). Strain 328 belongs to *S. coeruleorubidus* (*Actinomyces coeruleorubidus* Preobrazhenskaya) within the *Coeruleorubidus* group [12], which comprises several very similar species such as *A. coeruleorubidus*, *A. coeruleofuscus*, *S. steffisburgensis*, etc. The only feature characteristic of this group, but absent in strain 328, is a distinct green substrate mycelium pigment. Owing to this fact the identification of strain 328 as *S. coeruleorubidus* is only provisional.

The type strain of *Streptomyces finlayi* used in the present study was ATCC 23 340 (1 BUA 1869, International Streptomyces Project 5218, CBS 802.68).

Morphology and fine structure. Blocks of medium covered with sporulating aerial hyphae were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4 for 6 hr, subsequently in 2% osmium tetroxide at room temperature for 2 hr, followed by washing in 2% uranyl acetate for 8 hr. The blocks were then dehydrated in ethanol and embedded in Durcupan (Fluka). Ultra-thin sections were prepared and examined with electron microscope.

Whole-mount preparation of spores: coated grids were touched lightly to the surface of the sporulating aerial mycelium of the examined strains grown on oatmeal agar plates in Petri dishes; the material mounted on the grids was examined and photographed after drying at room temperature.

Whole-mount preparation and sections were examined and photographed in a UEMV-100B electron microscope at 75 kV.

Results

Fine structural changes during spore formation in strain FBUA 328 of *S. coeruleorubidus*. The spore surface structure of strain FBUA 328 was studied by electron microscopy of air-dried whole mounts and thin sections. The spores showed either spiny silhouettes or had a smooth surface in untreated whole mounts (Plate I, Figs 1, 2). The production of both spiny and smooth spores seems to be a constant characteristic of strain FBUA 328 although smooth spores are generally much less numerous than spiny ones. The spines, which are approximately 0.25 µm long and originate from the surface sheath, are either sharp and conspicuous, or thin, short and inconspicuous. A wide range of transitions was found in the surface ornamentation of spores, ranging from a smooth to a strongly developed superficial spiny layer with intermediate forms represented by a few thin, short and inconspicuous protrusions.

The sheath of the sterile stalk produces ornaments or rudiments only if, at the proximal end, the ultimate member of spore chain is also ornamented. If this is smooth, the stalk is also smooth over its whole length.

On the basis of the electron microscopic observations, smooth and differently ornamented spores are obviously not randomly distributed in the same spore chain, but have definite locations: mature smooth spores are almost exclusively localized near the sterile basal part of the hyphae (non-sporulating

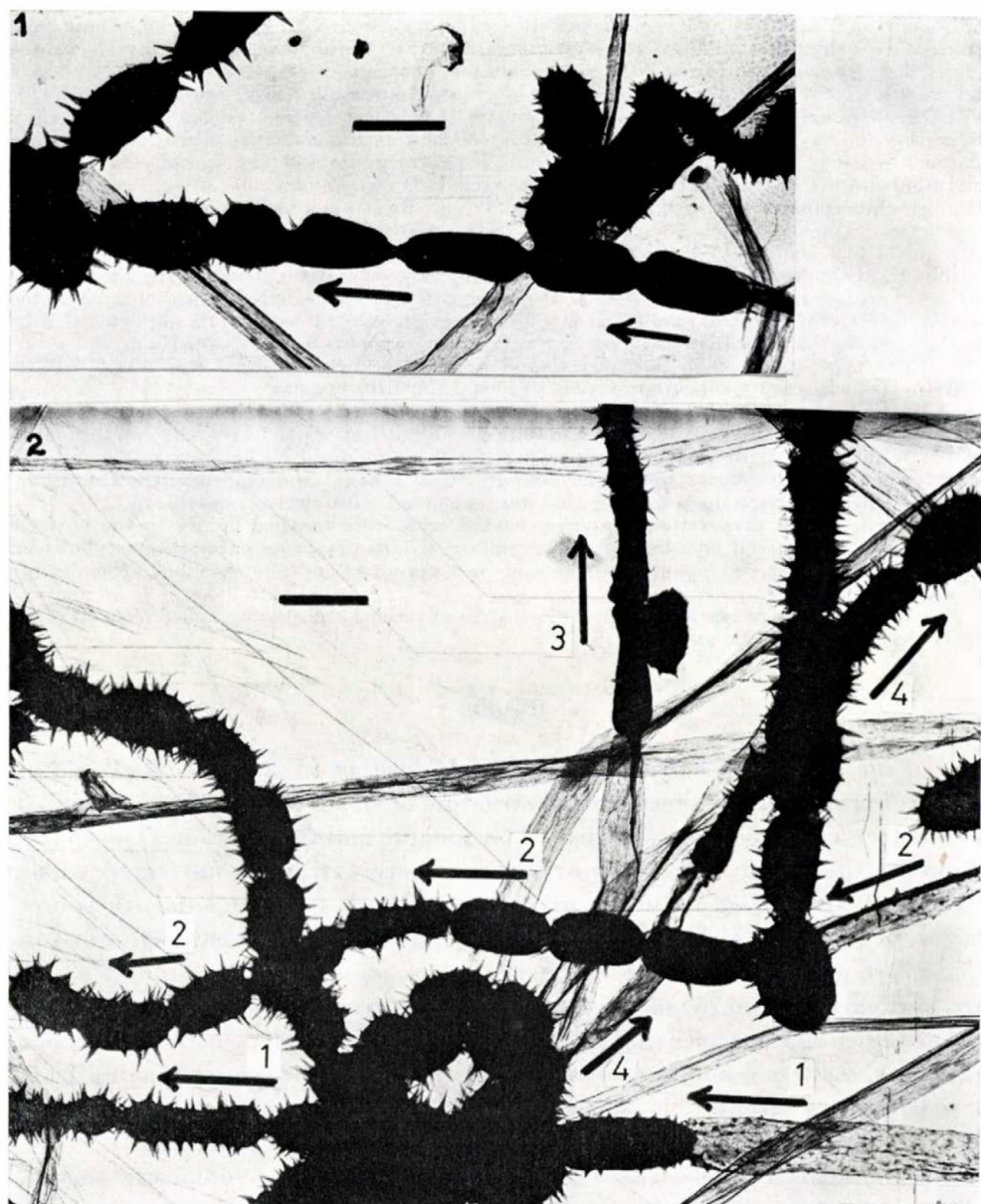


Plate I. Figs 1-2. *S. coeruleorubidus* (strain FBUA 328). Electron micrographs of 28-day-old culture on oatmeal agar (air dried whole mount). The spores show a wide range of transition in surface ornamentation from smooth ones through spores with a few thin, short and inconspicuous protrusions to spores with a strongly developed superficial spiny layer. Arrows indicate the direction from the sterile basal "stalk" towards the tip of the spore chain. The markers represent 1.0 μ m

"stalk" of sporophore) and are usually followed by spores with a few spines in the apex. At the distal end of the sporulating hypha, the last member of the spore chain is always the most conspicuously ornamented one. In other words, the density and size of spines is increasing from the sterile basal "stalk" of the sporulating hypha towards the tip of the sporulating distal region. Figures 1, 2 clearly show this particular pattern of surface ornamentation which seems obligatory in all such cases when heterogeneously ornamented spores are present in the same chain. The density of spiny ornaments on the surface of the individual members of the spore chain is evenly decreasing from the tip towards the non-ornamented smooth spore(s) carried by the adjacent "stalk" and the rate of decrease may be different in the different chains, but it is always regular. In many cases completely smooth spores are absent from the chain, only less ornamented ones occur nearby the "stalk". Plate II, Figs 3—7 and Plate III, Fig. 8 show electron micrographs of thin sections of *S. coeruleorubidus* spores. The detached surface layer (SL), which either produces spines or remains smooth even after maturation, is well-visible in thin sections of spores. Mature smooth spores covered with a thin intact sheath occur frequently in the cultures of strain FBUA 328.

Fine structural changes during spore formation in strain ATCC 23 340 of S. finlayi. The hairy ornamented spore chains are typical of strain ATCC 23 340, smooth spores occur only sporadically. Thorough observation of the spore chains containing smooth spores has shown that the particular pattern of surface morphology found with *S. coeruleorubidus* can be detected also in *S. finlayi*. Plates III and IV, Figs 9—11 show a spore chain in which only the first 8 spores are covered with "hairy" structures, while the subsequent members of the chain are completely smooth. Also, a gradual loss of ornamentation may be observed in as much as the density of appendages is decreasing from the first spore at the tip downward to the eighth member of the chain.

Discussion

According to WILDERMUTH [5, 6], structural differences between the surface "membranes" of non-sporulating (smooth) aerial hyphae and spores seems to be a general feature of *S. viridochromogenes* as well as of a number of other non-smooth spored streptomycetes. The events of spore formation are very probably synchronized in some way with the morphogenetic changes occurring in the sheath, and, as stated by WILDERMUTH [5], there is reason to conclude that unsegmented hyphae bearing spines or spine rudiments represent hyphae undergoing sporulation. The presence of smooth spores among spiny (or hairy) ones cannot be reconciled with the above statement about the synchrony of spore formation and morphogenesis of the sheath unless

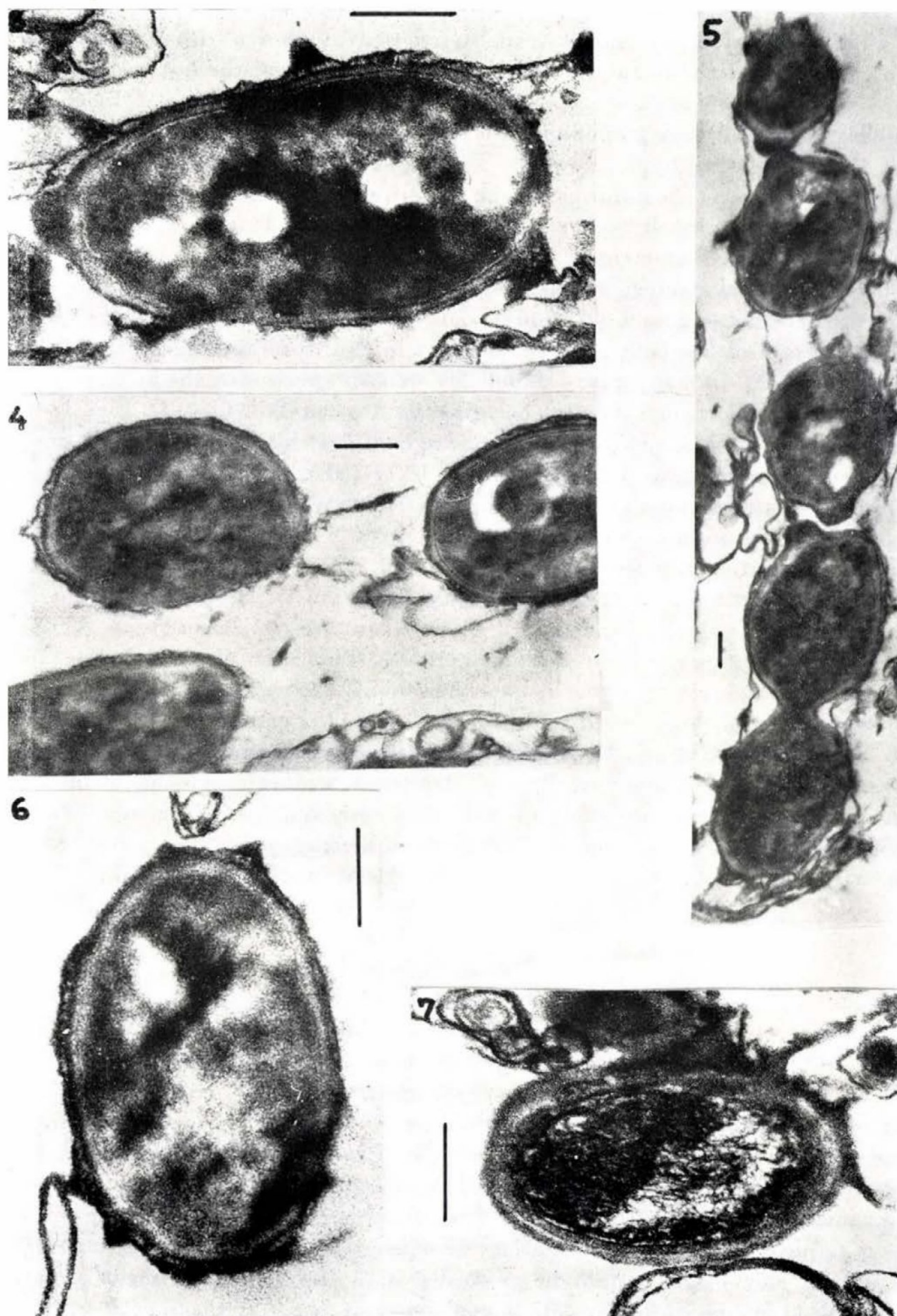


Plate II. Figs 3-7. Electron micrographs of thin sections of *S. coeruleorubidus* spores. The scale marker represents 0.25 μm . The detached surface layer may be smooth, or inconspicuously or strongly ornamented with spines around the spores

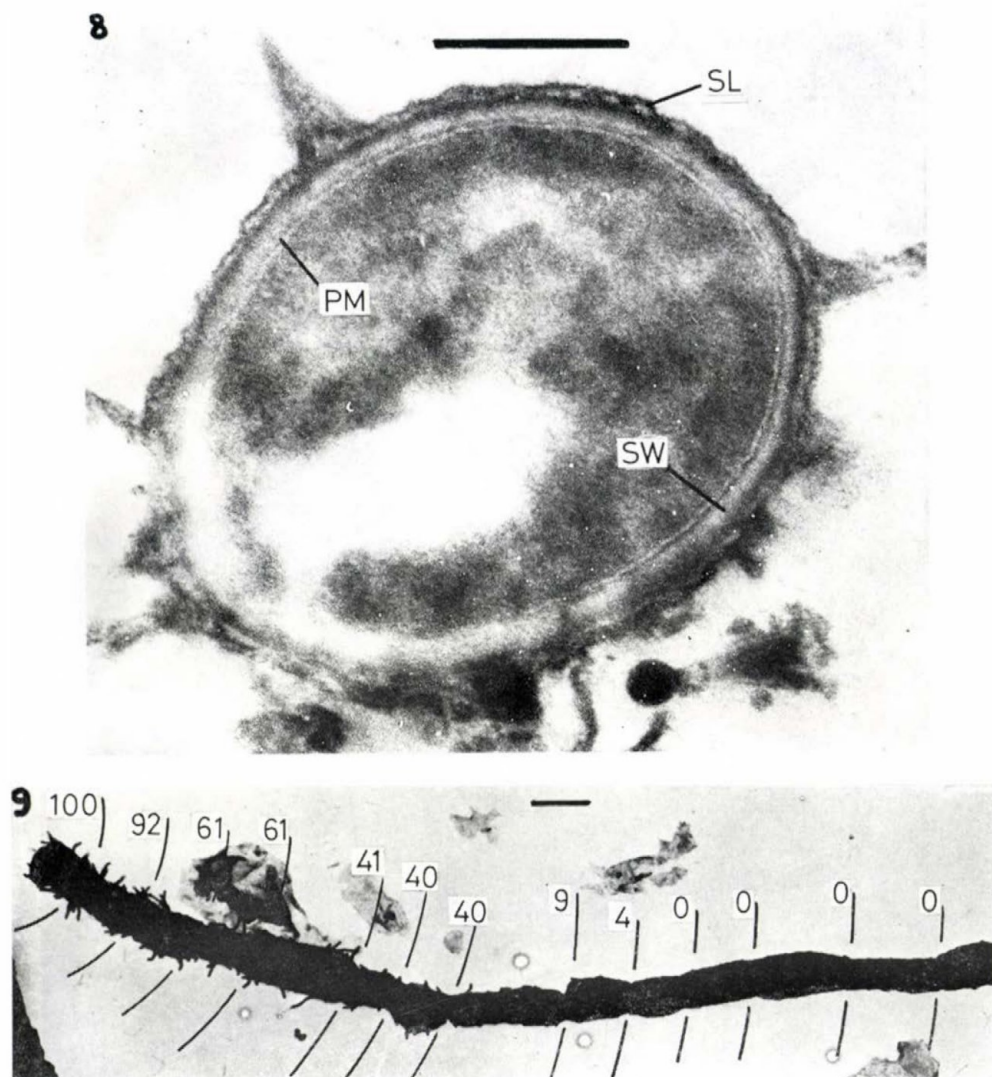


Plate III. Fig. 8. Electron micrograph of the thin section of a spore of *S. coeruleorubidus*. SL surface layer, SW spore wall, PM plasma membrane. Fig. 9. Silhouette of a spore chain (air-dried whole mount) of *S. finlayi*. The numbers indicate the relative density of hairy appendages on the surface of the individual spores taking the density of the most densely ornamented spore in the chain as 100%. The markers represent $0.25\ \mu\text{m}$ (upper photo) and $1.0\ \mu\text{m}$ (lower photo)

we suppose that smooth spores are naked, lacking the ornamented surface layer which was removed by disruption. But spores covered with a smooth or at least with a less ornamented surface sheath occur frequently in strain FBUA 328 and, presumably, less frequently also in *S. finlayi*. It is a fact that disturbances occurring in the morphological transformation of the

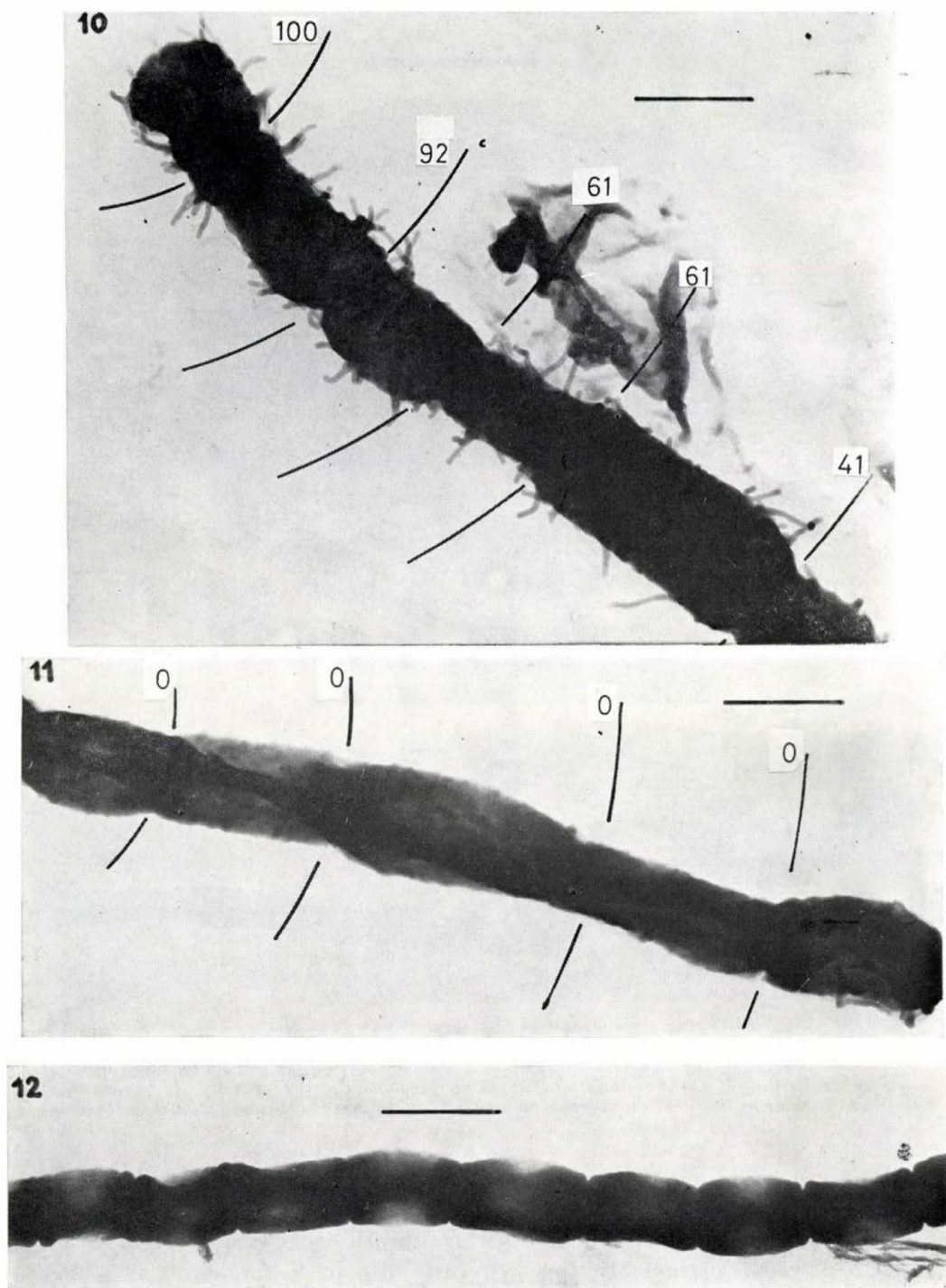


Plate IV. Figs 10–11. Magnified details of Fig. 9, showing hairy and smooth spores. The marker represents 1.0 μm . Fig. 12. Silhouette of a spore chain (air-dried whole mount) of *S. finlayi* var. *glaber* FBUA 584. The marker represents 1 μm .

surface layer (sheath) of a sporulating hypha are always associated with the appearance along the spore chain of a gradient in the intensity of surface ornamentation. In such spore chains the probability of the formation of spores with aberrant surface structures is increasing with the distance from the tip of the chain. These findings suggest that during sporulation, presumably still in the early stage, the cell unit at the tip, containing a dividing nucleoid, may produce substances which after translocation along the hyphae may induce a molecular re-arrangement of the surface sheath of the entire spore chain. If the function of this tip cell unit, which can be regarded as a "functional centre" is disturbed, a gradient depending on the measure of disturbance may arise along the hypha in the concentration of the substances responsible for the sheath's transformation and, accordingly, an adequate gradient is realized in the spores's surface ornamentation. Smooth and structured spores are being produced simultaneously by a few strains. The latter may be regarded as organisms having a certain tendency to produce deficiently functioning tip cell units at the division of the nuclear material in the sporulating aerial hyphae. In extreme cases the ability to produce such inducers may be lacking in the tip cell unit and the spore chain, the surface ornamentation of which is controlled by it, will remain completely smooth. Strains originating from such tip cell units are genetically deficient and grow and sporulate as smooth variants. Although we never observed completely smooth spore chains in the cultures of either *S. coeruleorubidus* or *S. finlayi*, such deficient spores might frequently occur in certain other species. From natural populations of *S. finlayi* we isolated [13] stable smooth variants which are known from the literature as *S. finlayi* var. *glaber* (Plate IV, Fig. 12).

On studying the growth kinetics, branch formation and cytological properties of *S. hygroscopicus*, SCHUMANN and BERGTER [14] suggested that the 50–100 μ m long hyphal section between the tip and the nearest branch may have a metabolism significantly different from that of the hyphal section behind the first branch. This critical hyphal section may be considered a "functional growth unit" presumably responsible for regulating the position of branches. These considerations call for research of the regulatory mechanisms operating in the segments of streptomycete hyphae.

Acknowledgements. The authors wish to thank Professor W. KURYLOVICZ (Poland), Dr. S. T. WILLIAMS (Great Britain) and Dr. H. PRAUSER (GDR) for inspiring discussions and helpful suggestions. Thanks are due to Miss ILONA TÓTH for valuable technical assistance.

REFERENCES

1. FLAIG, W., KÜSTER, E., BEUTELSPACHER, H.: Zbl. Bakt. II. Abt. Orig. **103**, 376 (1955).
2. ARAI, T., KURODA, S.: J. Bact. **83**, 924 (1962).
3. WILLIAMS, S. T., BRADSHAW, R. M., COSTERTON, J. W., FORGE, A.: J. gen. Microbiol. **72**, 249 (1972).
4. RANCOURT, M. W., LECHEVALIER, H. A.: Canad. J. Microbiol. **10**, 311 (1964).
5. WILDERMUTH, H.: Arch. Mikrobiol. **81**, 309 (1972).

6. WILDERMUTH, H.: Arch. Mikrobiol. **81**, 321 (1972).
7. SHIRLING, E. B., GOTTLIEB, D.: Int. J. syst. Bact. **16**, 313 (1966).
8. SZABÓ, I. M., MARTON, M.: Int. J. syst. Bact. **26**, 105 (1976).
9. CROSS, T., GOODFELLOW, M.: In SYKES, G., SKINNER, F. A. (eds): Actinomycetales: Characteristics and Practical Importance. Academic Press, New York 1973. p. 11.
10. WILLIAMS, S. T., HATFIELD, H. L., MAYFIELD, C. I.: In PRAUSER, H. (ed.): The Actinomycetales. Fischer, Jena 1970. p. 379.
11. SZABÓ, I. M., BUTI, I., IBRAHIM, A. N.: Zbl. Bakt. II. Abt. Orig. In press.
12. SZABÓ, I. M., MARTON, M., BUTI, I., FERNANDEZ, C.: Acta botan. Acad. Sci. hung. **21**, 387 (1975).
13. SZABÓ, I. M.: Microbial Communities in a Forest Rendzina Ecosystem. The Pattern of Microbial Communities. Akadémiai Kiadó, Budapest 1974.
14. SCHUMANN, E., BERGTER, F.: Z. allg. Mikrobiol. **16**, 201 (1976).

Address of the authors:

ISTVÁN M. SZABÓ
Department of Microbiology, L. Eötvös University
Múzeum krt. 4/a, H-1088 Budapest, Hungary

MÁRIA MARTON, ILONA BUTI
Research Institute for Soil Science and Agricultural Chemistry of the Hungarian Academy of Sciences,
Herman Ottó út 15, H-1022 Budapest, Hungary

LAJOS KONDICS
Department of General Zoology, L. Eötvös University
Puskin u. 3, H-1088 Budapest, Hungary

INFLUENCE OF HYPOTHERMIA ON THE GENERALIZED SHWARTZMAN REACTION

T. SZILÁGYI, S. TÓTH and G. LÉVAI

Institute of Pathophysiology and Institute of Anatomy, University Medical School, Debrecen

(Received November 12, 1976)

In normothermic rabbits the generalized Schwartzman reaction can usually be elicited by two intravenous injections of endotoxin spaced 24 hr apart. The reaction could be prevented by cooling the rabbits. Hypothermia is effective only at the time of the second injection. The protective effect can clearly be demonstrated by gross and histologic examination. The possible mechanism of the protective action of hypothermia is discussed.

In previous experiments it was shown that both general hypothermia [1] and local cooling [2] are effective in preventing the local Schwartzman reaction. The effect is particularly pronounced if the rabbits are hypothermic at the time of the provoking (second) injection. These observations led us to investigate the effect of hypothermia on the generalized Schwartzman reaction.

The classical generalized Schwartzman reaction is elicited by two intravenous injections of a large dose of endotoxin spaced 24 hr apart. Pathological changes such as fibrin and hyalin thrombi in the arterioles develop mainly in the kidneys, moreover they can be found in the adrenal glands, the lungs, the liver and other organs. The mechanism of the phenomenon is unclear. The most important problem to be solved is the cause underlying the disseminated intravascular coagulation occurring in the organs.

The present study was undertaken to test whether the generalized Schwartzman reaction was also prevented by hypothermia.

Materials and methods

Three-month-old rabbits weighing 1500 ± 200 g were used, as SMITH and THOMAS [3] found that unlike the local one, the generalized Schwartzman reaction is more likely to appear in young rabbits.

The rabbits were treated either with endotoxin prepared from *Escherichia coli* O111 strain according to Boivin or with Difco endotoxin (Lipopolysaccharide W. *E. coli* O55 : B5, Detroit, Michigan, U.S.A.). The single dose of the Boivin extract was the LD₅₀ dose of the preparation, while that of the Difco endotoxin was 200 µg. The generalized Schwartzman reaction was induced by two intravenous injections spaced 24 hr apart. Results were evaluated according to the renal cortical changes observed 24 hr after the second injection. For histological examinations, sections of the renal tissue were fixed in formalin or Susa's solution, embedded, cut and stained either with haematoxylin-eosin or with Endes's trichrome.

One group of rabbits was rendered hypothermic at the time of the second injection. Hypothermia was induced by means of plastic ice bag after superficial thiopental anaesthesia.

The provoking endotoxin injection was given after the body temperature of the animals had decreased to 28°C, where it was maintained for 1 to 1½ hr. Subsequently the animals were rewarmed; their body temperature reached the normal level in 3 to 6 hr.

Six rabbits were cooled at the time of the first endotoxin injection in the same way as described above. The animals were rewarmed after the injection and were normothermic at the time of the second injection, i.e. 24 hr later.

Results

Seventeen normothermic (control) rabbits survived after the second injection (9 treated with Boivin extract and 8 with the Difco preparation). Ten of the survivors (5 treated with Boivin extract and 5 with the Difco preparation) exhibited gross and microscopic changes characteristic of the generalized Shwartzman reaction.

In the group where hypothermia was induced at the time of the second injection, 13 rabbits (6 treated with Boivin extract and 7 with the Difco

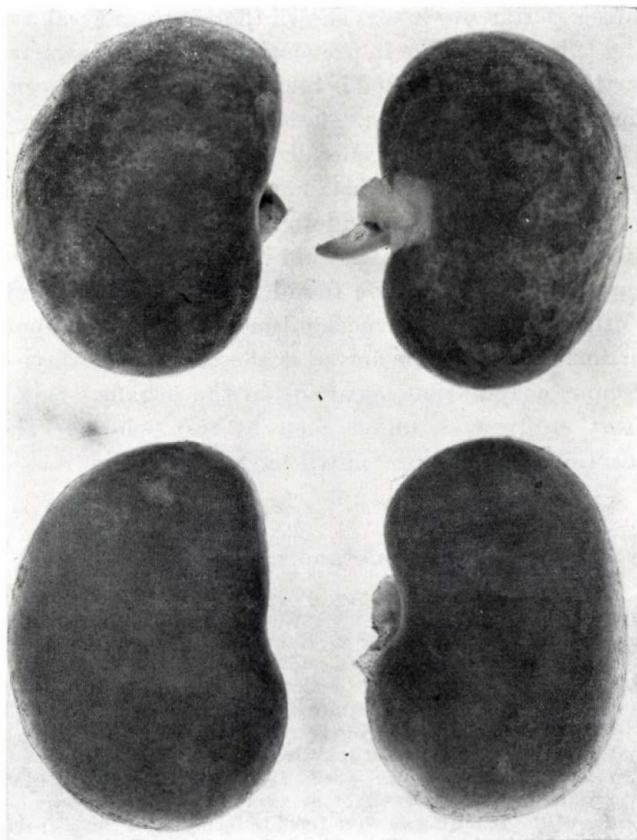


Fig. 1. Generalized Shwartzman reaction in rabbit kidney. Above: in normothermic animals. Below: in animals with a rectal temperature of 28°C

preparation) survived. In contrast to the normothermic (control) animals, none of these rabbits showed any sign of a generalized reaction, although they were rewarmed after the injection and were observed for several days.

There was a striking difference in the gross appearance of the kidneys between the normothermic and hypothermic animals, as seen in Fig. 1.

In most of the normothermic rabbits, histological examination revealed characteristic severe renal cortical necrosis after the second endotoxin injection. Fig. 2 shows the grave change of the tubular epithelium with haemorrhage.

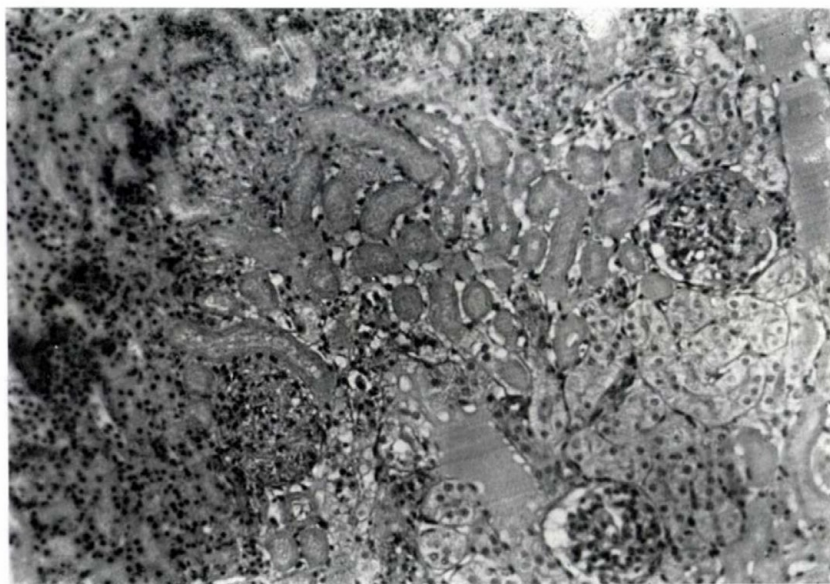


Fig. 2. Generalized Schwartzman reaction in normothermic rabbits. Necrosis of tubular epithelial cells, swollen glomeruli. Interstitial haemorrhagic reaction at the periphery of the necrotic area. Haematoxylin-eosin staining, $\times 180$

ges. Fig. 3 shows that the vessels of the renal cortex were thrombosed and characteristic fibrinoid necrosis developed in the glomeruli (Fig. 4).

In contrast, no gross or microscopic evidence of a renal lesion could be detected in rabbits that were hypothermic at the time of the second endotoxin injection. Fig. 5 shows intact glomeruli and normal structure of the tubular cells.

Four of the six rabbits that were hypothermic at the time of the first endotoxin injection developed serious renal lesions, similar to those seen in the normothermic (control) animals.

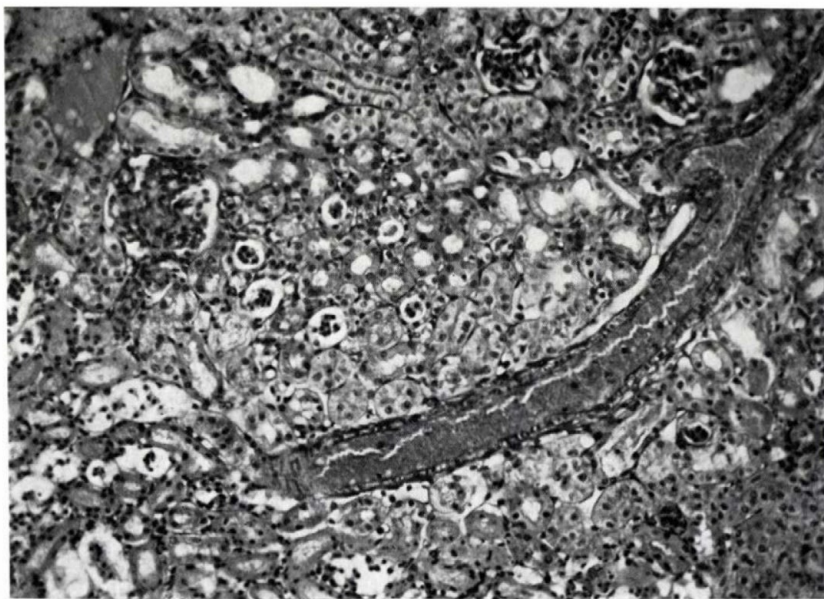


Fig. 3. Generalized Schwartzman reaction in normothermic rabbits. Occluded renal vessels. Haematoxylin-eosin staining, $\times 180$

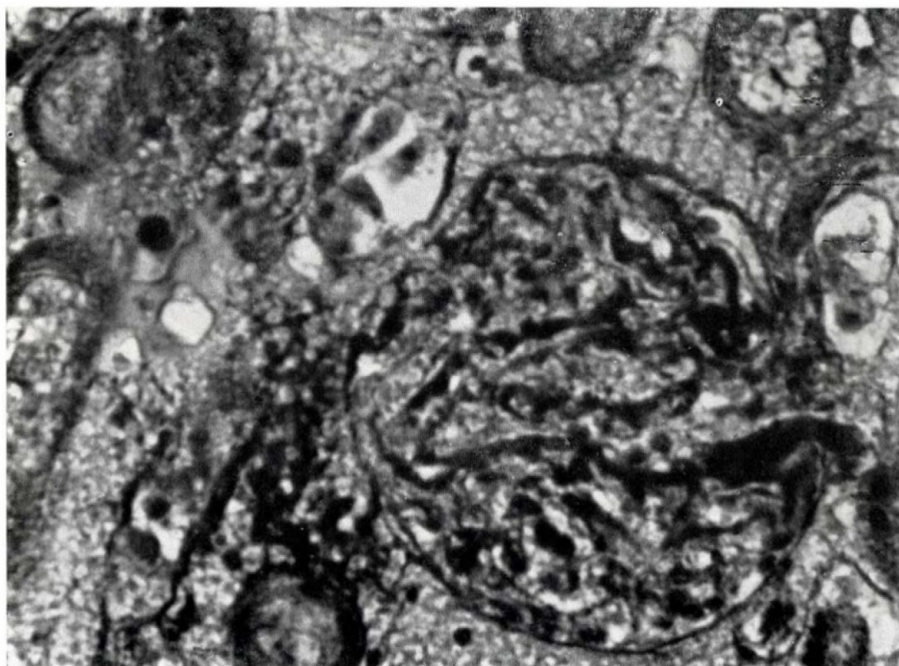


Fig. 4. Generalized Schwartzman reaction in normothermic rabbits. Fibrinoid necrosis in glomeruli. Endes's trichrome staining, $\times 450$

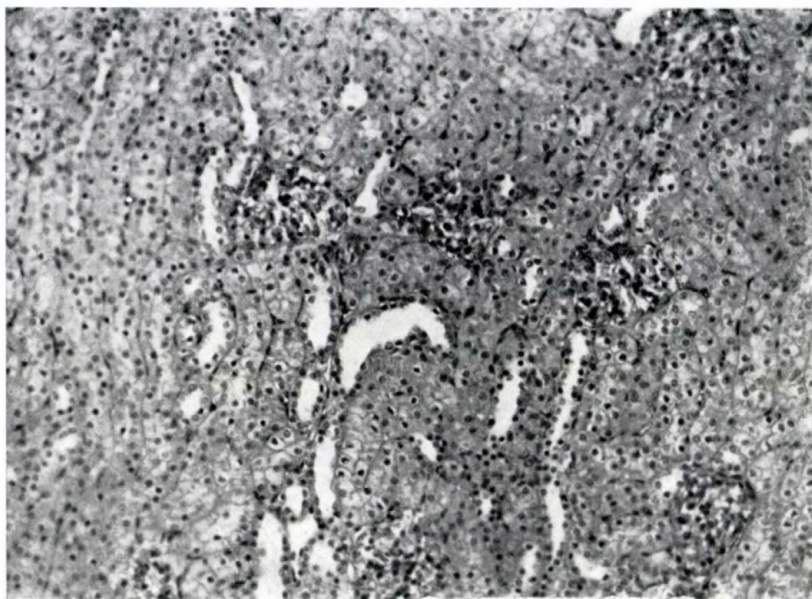


Fig. 5. Generalized Shwartzman reaction in rabbits with a rectal temperature of 28°C. Intact glomeruli, normal structure of tubular cells, moderate oedema. Haematoxylin-eosin staining, $\times 180$

Discussion

The present experiments show that while the generalized Shwartzman reaction can be induced by two intravenous injections of endotoxin in the majority of normothermic rabbits, the reaction can be prevented by cooling the animals. Hypothermia is effective only if it is induced at the time of the second injection. The protective effect was seen clearly both on gross and microscopic examination. In previous experiments [1, 2] it was shown that the local Shwartzman reaction was prevented by general or local cooling. The present results indicate that, like in experiments on anaphylaxis [4], systemic lesions are also prevented by general hypothermia.

As to the mechanism of this prevention, several factors may be involved in it. It was reported that the reduction of glomerular filtration prevents the development of serious renal lesions [5]. It is therefore of interest that, according to VÉCHELYI and HÁRSING [6] renal circulation and glomerular filtration are considerably reduced at body temperatures below 30°C. The experimental results of HORN and COLLINS [7], and those of GAZZANIGA and O'CONNOR [8] suggest that PMN leukocytes and the lysosomal enzymes released from these cells may be of crucial importance in the development of the generalized Shwartzman reaction, and we could show that the release of lysosomal enzymes was greatly prevented by hypothermia [9].

WHITAKER *et al.* [10] suggested that adrenaline and alpha-receptors played an important role in the mechanism of the generalized Shwartzman reaction. Besides, we found that two intravenous injections of endotoxin resulted in the increase of catecholamine sensitivity, a change which might be of some importance in inducing the reaction. In hypothermic animals, however, catecholamine sensitivity was affected neither by the first nor by the second endotoxin injection [11].

The above findings suggest that the mechanisms operative in the generalized Shwartzman reaction may partly or completely be derepressed in hypothermic animals.

REFERENCES

1. SZILÁGYI, T., KOCSÁR, L., CSERNYÁNSZKY, H.: *Acta microbiol. Acad. Sci. hung.* **3**, 327 (1956).
2. SZILÁGYI, T., TÓTH, S., DESEŐ, G., BENKŐ, K.: *Beitr. path. Anat.* **153**, 395 (1974).
3. SMITH, R. T., THOMAS, L.: *Proc. Soc. exp. Biol. (N. Y.)* **86**, 806 (1954).
4. SZILÁGYI, T., KOCSÁR, L., GYULAI, F.: *Acta physiol. Acad. Sci. hung.* **8**, 393 (1955).
5. KLASSEN, J., MILGROM, F.: *Proc. Soc. exp. Biol. (N. Y.)* **134**, 980 (1970).
6. VÉGHÉLYI, P. V., HÁRSING, L.: *Klin. Wschr.* **33**, 908 (1955).
7. HORN, R. G., COLLINS, R. D.: *Lab. Invest.* **18**, 101 (1968).
8. GAZZANIGA, A. B., O'CONNOR, W. E.: *Ann. Surg.* **172**, 804 (1970).
9. SZILÁGYI, T., TÓTH, S., CSERNYÁNSZKY, H., SÜMEGI, J.: *Agents and Actions* **6**, 721 (1976).
10. WHITAKER, A. N., MCKAY, D. G., CSÁVOSSY, I.: *J. Path.* **56**, 153 (1969).
11. CSÁKÓ, G., SZILÁGYI, T., CSERNYÁNSZKY, H., TÓTH, S.: *Med. Biol. (Helsinki)* **54**, 243 (1976).

Address of the authors:

TIBOR SZILÁGYI
Institute of Pathophysiology, University Medical School,
H-4012 Debrecen, P.O.B. 23, Hungary

SÁNDOR TÓTH
State Hospital,
H-3244 Parádafürdő, Hungary

GÉZA LÉVAI
Institute of Anatomy, University Medical School,
H-4012 Debrecen, P.O.B. 14, Hungary

SURVEILLANCE OF R-PLASMIDS*

H. RISCHÉ, H. TSCHÄPE and W. WITTE

Institute of Experimental Epidemiology, Wernigerode, G.D.R.

(Received November 22, 1976)

The surveillance of R-plasmids consists of: (1.) Ecological and epidemiological investigations. (1.1.) Prevalence of R-plasmids in pathogenic and apathogenic bacteria occurring in the biotic environment (man, animal); in the abiotic environment (sewage, food, feed); under high selection pressure (hospital, animal production, plant production). (2.) Biological investigations. (2.1.) Genetic properties of R-plasmids: (2.2.) Plasmid induced variations of the properties of the host bacteria (virulence, phage pattern, antigenic pattern, serological properties). The problems resulting from plasmid-mediated drug resistance call for a strict chemotherapeutic drug policy with the regard to ecological processes.

Chemotherapy and, to a limited extent, chemoprophylaxis of bacterial infections and communicable diseases of human beings, animals, and to an increasing extent also of plants, has become imperative for reasons of health and economy. The large-scale application of the same drugs to nutritive purposes in livestock breeding is also an important field [1–6].

Soon after the discovery and development of antimicrobial drugs (antibiotics) it was realized that populations of bacteria showing sensitivity to certain drugs were able to produce drug-resistant variants. Subsequent analyses of this phenomenon showed that extra-chromosomal elements, drug-resistance plasmids (R-factors, R-plasmids) were the responsible factor [7, 8]. In this connection, chromosomal resistance is hardly of any importance. Mostly through enzymes the R-plasmids impart resistance to one or more (up to ten) antibiotics simultaneously. In addition, R-plasmids also determine the ability of conjugation spreading to other non-plasmid containing strains (infectious nature of plasmids), which may belong to other species, families or even orders of the Gram-negative bacteria [7, 8].

So, e.g., a transfer of the plasmids from *Escherichia coli* to *Shigella sonnei*, *Salmonella*, *Pseudomonas* etc. or *vice versa* is possible. Gram-positive cocci differ from Gram-negative bacteria in that an intergeneric plasmid-transfer has not been observed in them.

Experimental trials have, however, demonstrated that staphylococcus resistance plasmids can be transferred by the mixed growth of donor and

* Presented at the 7th Congress of the Hungarian Society of Microbiology, Budapest September 6–9, 1976.

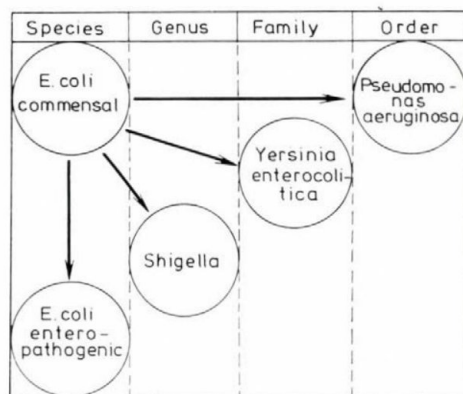


Fig. 1. Transfer of R-plasmids

recipient cells. This transfer also occurs from *Staphylococcus epidermidis* to *Staphylococcus aureus* and is probably not due to transduction; it requires a cell-to-cell contact [9].

As these strains occur ubiquitously, *S. epidermidis* is presumed to represent a reservoir for resistance-plasmids.

Other plasmid-governed properties are also known; these properties are the formation of enterotoxins, haemolysins and H_2S , the fermentation of lactose, raffinose, and saccharose as well as changes of antigens and phage types [10–12].

Enrichment of plasmid-carrying bacteria occurs in regions with high selection pressure. For the R-plasmids these are groups of human beings, animals or plants that are given therapeutic, prophylactic or nutritive drugs. It is from these regions that the infectious drug resistance is transmitted directly or indirectly to other regions and populations. It is necessary to note that theoretically two different mechanisms have to be distinguished, which under natural conditions are frequently interlaced [12–15]. These are (i) the process of conjugation between plasmid-carrying and non-plasmid-carrying strains (transfer, infectious nature), and (ii) the direct or indirect transmission of R-plasmid-carrying bacteria.

These processes lead to the spread of the infectious resistance into the biotic environment (human being, animals, plants) and the abiotic environment (milk, food, feed, sewage) even without actual selection pressure. In *S. aureus* this transmission of resistant cells between different ecological spheres is of limited significance.

Based on ecological-epidemiological investigations on the spreading and the main ways of transmission of infectious drug resistance using more than 10 000 samples during the last seven years, we have proposed two models. They are shown in Figs 2 and 3 [7].

Fig. 2 demonstrates the way of transmission of infectious drug resistance from animal to man. R^+ bacteria (e.g. *E. coli*) of the intestinal flora are excreted with faeces. They reach human beings either directly (contamination) or indirectly through victuals (especially milk) or through sewage contaminated victuals. In the sewage R^+ *E. coli* strains of animal origin may come into contact with R^- *E. coli* strains from man and animals. They exchange their R-plasmids. If these R^+ *E. coli* strains reach human beings, they are directly integrated into the intestinal flora by colonization or they transmit their R-plasmids to the resident intestinal flora by intestinal transfer.

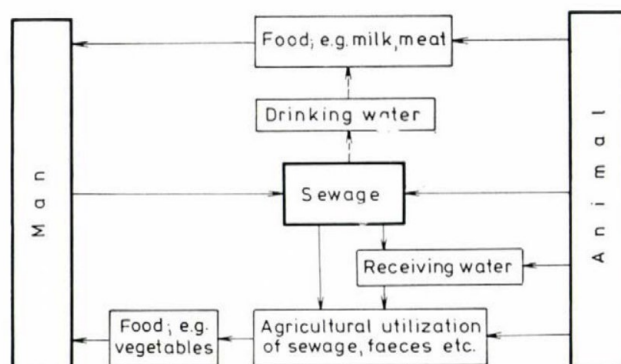


Fig. 2. Transmission of R^+ bacteria from animal to man

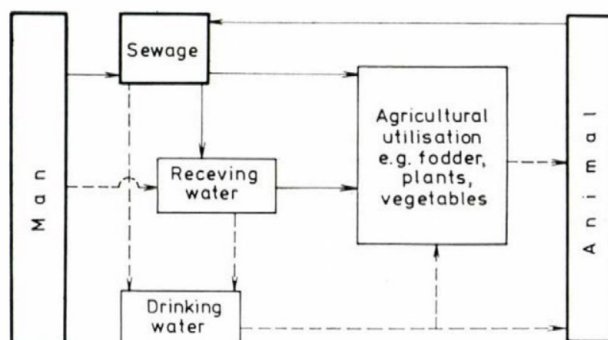


Fig. 3. Transmission of R^+ bacteria from man to animal

Similarly, the way from man to animal goes mainly through water and feed contaminated by sewage (Fig. 3).

In *S. aureus* there are different host-specific varieties [16, 17] characteristic of certain animal species. Colonization or infection of a host-specific variety in an extraneous host is infrequent. So there is no danger of a spreading of R^+ *S. aureus* from animals to man. In a chicken farm e.g. we found multi-

resistant *S. aureus* strains from synovitis. These strains differ from strains of human origin by their biochemical properties. In nasal swabs and furuncles from men working in this chicken farm we found strains of the variety *hominis*, which were seldom resistant to penicillin while sensitive to all other drugs.

The ways of transmission from and to plants will not be dealt with.

In accordance with other authors we assume that (i) the ubiquitous transmission of R-plasmids among enterobacteriaceae and probably among all Gram-negative bacteria; (ii) the steadily increasing incidence of R-plasmid-containing bacteria in the biotic and abiotic environment; and (iii) the incidence of new resistance determinants to recently developed drugs jeopardize the chemotherapy of bacterial infectious diseases and will have incalculable consequences [6, 15, 18]. Thus it is necessary to pursue a national antibiotics policy, which is internationally co-ordinated and the essential parts of which must contain ecological-epidemiological surveillance and drug monitoring programmes [5, 7, 19, 20]. As a result of our ecological-epidemiological investigations the following points have been considered necessary in the surveillance of infectious drug resistance.

1. Surveillance of the prevalence and the genetic properties of R-plasmids and other plasmids in pathogenic bacteria, using phage typing and other epidemiological laboratory methods to differentiate these bacteria.

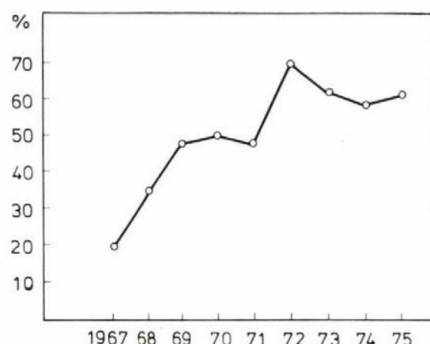


Fig. 4. Resistance of *S. sonnei* in the G.D.R. (1967-1975)

Surveillance of the prevalence and examination of R-plasmid types of the most important pathogenic enterobacteria in the G.D.R. — *S. sonnei* — showed e.g. an increase (Fig. 4) during the last nine years (>1000 strains/year). The increase of tetracycline single resistance is interesting, as in the G.D.R. tetracycline is not used against shigellosis and *S. sonnei* is a bacterium limited to human beings. These results, but particularly those concerning river-water [21] and receiving water which had not been contaminated by sewage or faeces of human beings, indicated that the tetracycline single resistance was

mainly due to feeding animals with this antibiotic. Also in staphylococci, the effect of selection pressure on the development of drug-resistance is pronounced. Multiresistant strains harbouring several different resistance plasmids occur mainly in hospitals and in farms of industrial animal production. *S. aureus* strains from hospital infections differ from strains of non-hospital origin. They are less sensitive to antibiotics, more frequently resistant to penicillin and often multiresistant (Fig. 5).

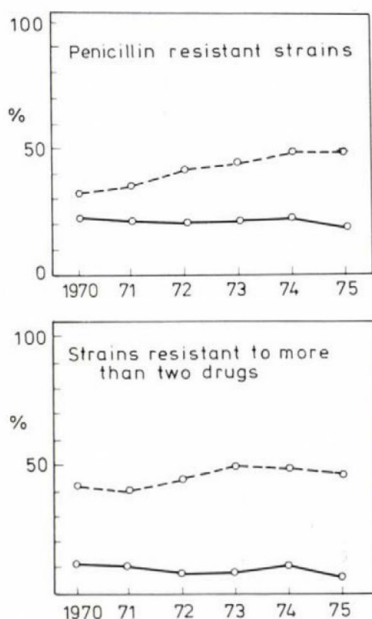


Fig. 5. Resistance of *S. aureus* in and outside hospitals; ○——○ strains of different origin, ○——○ strains from hospital infections

Obviously, there is no remarkable resistance development outside the hospital though strains from hospitals can be distributed outside [22]. This can be explained by the *in vivo* loss of resistance plasmids from *S. aureus* cells in the absence of selection pressure [23].

2. Surveillance of the prevalence and genetic properties of R-plasmids and other plasmids of the intestinal bacteria from communities under different selection pressures (hospital, healthy urban and rural population, workers in the antibiotics industry, industrial animal and plant production).

Fig. 6 demonstrates the prevalence of R⁺ bacteria in the biotic environment; it shows that human beings and animals without selection pressure have about 50%; whereas animals receiving nutritive doses of tetracycline have 100% tetracycline single-resistance plasmids. Under the selection pressure of

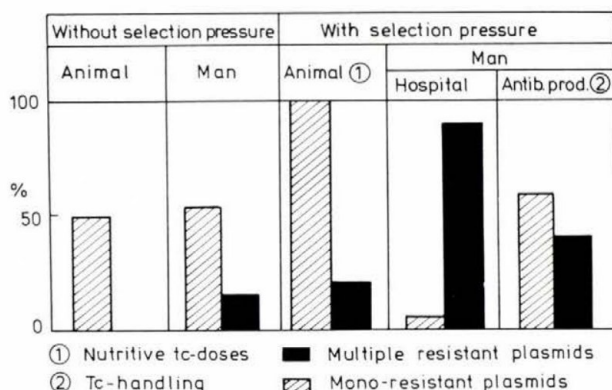


Fig. 6. Prevalence of R^+ bacteria in man and animal with and without drug pressure
a large assortment of antibiotics multiple drug-resistant plasmids with partly rare resistance determinants prevail in hospitals.

3. Surveillance of the prevalence and of the genetic properties of R-plasmids and other plasmids among bacteria in sewage, river water, and in the soil.

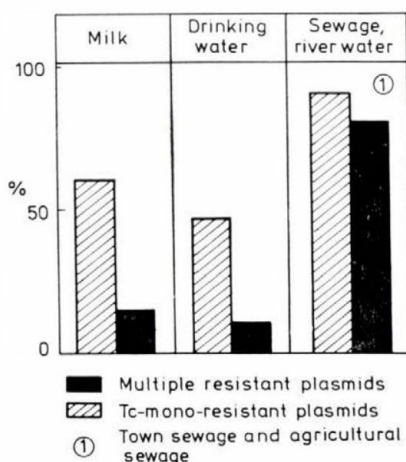


Fig. 7. Prevalence of R^+ bacteria in the abiotic environment

Fig. 7 demonstrates the prevalence of R^+ bacteria in the abiotic environment. Sewage is important as the place of R-plasmid exchange and as R^+ bacteria reservoir; milk serves as an essential vehicle for the transmission of R^+ bacteria to human beings [3, 6, 7, 15, 24].

4. Surveillance of the prevalence and genetic properties of R-plasmids and other plasmids in bacteria from food and feed.

The surveillance programme is part of the antibiotic policy. It further includes the surveillance of the efficiency of drugs in the various fields of application in medicine, veterinary medicine, and animal feeding (drug monitoring).

In addition to the interpretation of the WHO and some authors (6, 18, 25] we should propose not only a bipartition but a tripartition of the drugs with regard to their fields of application [7].

1. Drugs for human therapy and only exceptionally prophylactic.
2. Drugs for the therapy and prophylaxis of animals.
3. Drugs for paratherapeutic application (animal feeding, plant protection).

The antibiotics policy should have the following aims:

- estimation and prognosis of the use of drugs in the main fields of application;
- limitation of the spread of R-plasmids;
- planning of drug production;
- development of different drugs for specific application.

These aims are component parts of the antibiotics policy, a summary of which is seen in Table I [7]. In the G.D.R. such a joint antibiotics policy is under organization.

Table I

Chemotherapy policy

1. <i>Surveillance of infectious drug resistance</i>	2. <i>Drug monitoring</i>
1.1. Ecological and epidemiological investigations (surveillance programme)	2.1. Effectiveness of the drugs
1.1.1. Prevalence of R-plasmids in pathogenic and apathogenic bacteria, occurring	2.1.1. in therapy and prophylaxis of man
— in the biotic environment (man, animal)	2.1.2. in therapy and prophylaxis of animals
— in the abiotic environment (sewage, drinking water, food, feed)	2.1.3. in animal production (nutritive doses)
— under high selection pressure (hospital, animal production, plant production, antibiotic industry)	2.1.4. in plant production
1.2. Biological investigations	2.2. Economy in the use of drugs
1.2.1. Genetic properties of R-plasmids	2.3. Side effects of drugs
1.2.2. Plasmid induced variations in the properties of host bacteria (virulence, phage pattern, antigen pattern, serological properties)	
3. <i>Aim of the chemotherapy policy</i>	
3.1. Estimation and prognosis of the use of chemotherapy in	
3.1.1. therapy of man	
3.1.2. therapy of animals	
3.1.3. prophylaxis of animals	
3.1.4. animal production	
3.1.5. plant production	
3.1.6. other paratherapeutic fields of application (feed industry, cosmetics)	
3.2. Methods for limitation of the spreading of infectious drug resistance	
3.3. Production and import of antibiotics	
3.4. Development of new drugs for human medicine, veterinarian medicine and other branches	

REFERENCES

1. BURKE, J. F.: Ann. Rev. Med. **24**, 284 (1973).
2. HOPKINS, H. C.: FDA papers **6**, 14 (1972).
3. HUBER, W. G.: J. pure appl. Chem. **21**, 377 (1970).
4. JUKES, T. H.: BioScience **22**, 526 (1972).
5. KUNIN, C. M., TUPASI, T., CRAIG, W. A.: Ann. intern. Med. **79**, 555 (1973).
6. Report to the Commissioner of the FDA Task Force on the Use of Antibiotics. U.S. Dept. HEW, PHS, FDA, Rockville, Md. 1972.
7. TSCHÄPE, H., RISCHE, H.: Ökologie und epidemiologische Bedeutung der infektiösen Chemotherapeutika-Resistenz. Beitr. Hyg. Epidemiol. Bd. 22, Joh. Ambr. Barth, Leipzig 1974.
8. WATANABE, T.: Curr. Topics Microbiol. **56**, 49 (1972).
9. WITTE, W.: Transfer of Drug-resistance Plasmids in Staphylococci. In preparation.
10. RISCHE, H., TSCHÄPE, H.: Z. ges. Hyg. **20**, 8 (1974).
11. SMITH, H. W., LINGOOD, M. A.: J. med. Microbiol. **4**, 467 (1971).
12. TSCHÄPE, H., RISCHE, H.: Z. allg. Mikrobiol. **14**, 337 (1974).
13. KEMP, G., KISER, J.: J. animal Sci. **31**, 1107 (1970).
14. MERCER, H. D., POCURULL, H. D., GAINES, S., WILSON, S., BENNET, J. V.: Appl. Microbiol. **22**, 700 (1971).
15. RICHMOND, M. H.: J. appl. Bact. **35**, 155 (1972).
16. MEYER, W., J. system. Bact. **17**, 387 (1967).
17. WITTE, W., GRIGOROWA, M., BAJLJOSOV, D., HUMMEL, R.: On the Ecology of Staphylococcus aureus: Comparative Characterization of Staphylococcus aureus Strains Isolated from Man, Cattle and Sheep in Bulgaria and in the G.D.R. In preparation.
18. Swann-Report: Joint Committee on the Use of Antibiotics in Animal, Husbandry and Veterinary Medicine. H. M. S. O., London 1969.
19. KLEIN, U., GIKALOV, J., KELLER, M., HOIGNE, R.: Schweiz. med. Wschr. **102**, 1083 (1972).
20. RISCHE, H.: Z. Militärmed. **3**, 325 (1972).
21. SCHULZE, L.: Dissertation, Magdeburg 1976.
22. GRÜN, L.: Staphylococcus aureus in Klinik und Praxis. Wissenschaftliche Verlagsgesellschaft, Stuttgart 1964.
23. WITTE, W., KÜHN, H.: Z. allg. Mikrobiol. **16**, 563 (1976).
24. FRITZ, J. H.: J. publ. Hlth **62**, 414 (1972).
25. WHO Technical Report Ser. No. 430, 1969.

Address of the authors:

HELMUT RISCHE, HELMUT TSCHÄPE, WOLFGANG WITTE
 Institut für Experimentelle Epidemiologie
 DDR-37 Wernigerode, Burgstrasse 37, German Democratic Republic

NOTE

INDOLE POSITIVE VARIANT OF SHIGELLA BOYDII 1

MARIA M. ÁDÁM, CATHERINE KOVÁCH, L. KEREKES and ROSE BÉRY

National Institute of Hygiene, Budapest and Public Health Station, Eger

(Received October 18, 1976)

In a home for mentally handicapped children indole positive variants of *Shigella boydii* 1 were isolated beside indole negative strains of the same serotype. The variants differed from the indole negative counterparts in fermenting dulcitol, raffinose, and in the absence of splitting trehalose. In antigenic structure the indole positive variant was identical with the type strain. The isolates gave positive guinea pig eye test.

In a home for mentally deficient children in the Hungarian town Eger, in February, 1976, a typical indole negative *Shigella boydii* 1 strain was isolated from a person with enteritis. After a month the same organism was isolated from the same person (strain 5244). Some days later an indole-producing *S. boydii* 1 strain was cultured from a child with enteric symptoms (strain 8135). The indole positive variant was again recovered from the same child and was also isolated at environmental screening from a healthy boy (strains 24 833 and 25 640). A further indole-producing strain was isolated from another healthy child (strain 26 478).

Bacterial strains and methods. Isolations were made on desoxycholate-citrate agar [1] and the cultures were maintained in agar stabs [1]. As type cultures, strains of the Hungarian National Collection of Medical Bacteria were used.

Biochemical and serological methods were as described earlier [1–4].

Examination of patients' sera was performed with passive haemagglutination, using formalized sheep erythrocytes sensitized with the supernatant of 1 hr boiled saline suspensions of bacteria. Dilution of sera was made in tubes and testing was performed on TAKÁTSY's Microtitrator plates [5].

Guinea pig eye test was performed and evaluated as described by SERÉNY [6].

Results. Biochemical reactions of the indole-producing *S. boydii* 1 cultures were characteristic of shigellae. Unlike indole negative cultures, which did not ferment the following substrates, indole positive strains gave positive reactions with dulcitol (2 days), and raffinose (2–4 days). D-galactose was delayed positive (3–5 days), while indole negative strains fermented this sugar promptly. Indole positive isolates gave negative trehalose test, whereas indole negative strains were trehalose positive.

Antigenic analysis by cross absorption reaction showed that the indole negative type strain was serologically identical with the indole positive culture.

The guinea pig eye test was positive with all *S. boydii* 1 isolates.

Haemagglutination titres in patients and healthy children did not differ significantly from that of the average titre of 50 healthy adults.

In the same period *S. sonnei*, *S. flexneri* and *Escherichia coli* O124 were isolated beside *S. boydii* 1. The patients and healthy children showed no significant levels of *E. coli* O124 antibodies.

Discussion. *S. boydii* 1 strains have been described by EDWARDS and EWING as being indole negative in 100% [2]. Indole positive, dulcitol fermenting *S. boydii* 1 strains were first isolated by ALDOVÁ et al. [7] from a healthy woman.

The finding of biochemical variants is of taxonomical and genetic interest. As in our case at first a biochemically typical (indole negative) strain was isolated, it is supposed that the circulating type had acquired new biochemical characteristics.

Acknowledgement. The authors are indebted to Dr. B. ROWE (Salmonella and Shigella Reference Laboratory, Central Public Health Laboratory, London) for checking the strains, to Miss MARIA LUDÁNYI, Miss ÉVA TÖRÖK (Budapest) and Miss ÉVA VIZI (Eger) for skilled technical assistance, and for collecting patients sera.

REFERENCES

1. Módszertani Útmutató, National Institute of Hygiene, Budapest 1969.
2. EDWARDS, P. R., EWING, W. H.: Identification of Enterobacteriaceae. Burgess, Minneapolis 1974.
3. KAUFFMANN, F.: The Bacteriology of Enterobacteriaceae. Munksgaard, Copenhagen 1966.
4. LE MINOR, L.: Le diagnostic de laboratoire des bacilles à Gram négatif entérobactéries. La Tourelle, Saint-Mandé 1972.
5. TAKÁTSY, GY.: Acta microbiol. Acad. Sci. hung. **3**, 191 (1955).
6. SERÉNYI, B.: Acta microbiol. Acad. Sci. hung. **4**, 367 (1957).
7. ALDOVÁ, E., LÁZNIČKOVÁ, K., HAUSNEROVÁ, S.: Zbl. Bakt. I. Abt. Orig. **215**, 274 (1970).

Address of the authors:

MARIA M. ÁDÁM, LÁSZLÓ KEREKES
National Institute of Hygiene
H-1966 Budapest, P.O.B. 64, Hungary

CATHERINE KOVÁCH, ROSE BÉRY
Public Health Station
Széchenyi u. 27, H-3300 Eger, Hungary

INDEX

<i>Karasseva, V., Sziklai, I., Ördögh, M.</i> : Quantitation of Seven Elements in <i>Mycobacterium phlei</i> and <i>Mycobacterium bovis</i> by Neutron Activation Analysis	175
<i>Ádám, É., Nász, I., Medveczky, P.</i> : Comparative Studies on the Soluble Proteins of Adenovirus Type 1	181
<i>Nagy, G., Mezey, I.</i> : The Use of Ion Exchange Chromatography for Demonstration of Rubella-Specific IgM Antibodies	189
<i>Cameron, J., Stainer, D. W.</i> : Pertussis Vaccine — Fact and Fiction	195
<i>Bergmann, P., Mebel, S., Rustenbach, S.</i> : Morphology of <i>Salmonella typhi</i> in Different Inactivation Techniques	207
<i>Polotsky, Yu. E., Dragunskaya, E. M., Seliverstova, V. G., Avdeeva, T. A., Chakhutinskaya, M. G., Kétyi, I., Vertényi, A., Ralovich, B., Emödy, L., Málovics, I., Safonova, N. V., Snigirevskaya, E. S., Karyagina, E. I.</i> : Pathogenic Effect of Enterotoxigenic <i>Escherichia coli</i> and <i>Escherichia coli</i> Causing Infantile Diarrhoea	221
<i>Szabó, I. M., Kondics, L., Marton, M., Buti, I.</i> : Changes in the Surface Layer (Sheath) of the Cell Wall of Streptomyces During Sporulation	237
<i>Szilágyi, T., Tóth, S., Lévai, G.</i> : Influence of Hypothermia on the Generalized Schwartzman Reaction	247
<i>Rische, H., Tschäpe, H., Witte, W.</i> : Surveillance of R-Plasmids	253
<i>M. Ádám, M., Kovách, C., Kerekes, L., Béry, R.</i> : Note. Indole Positive Variant of <i>Shigella boydii</i> 1	261

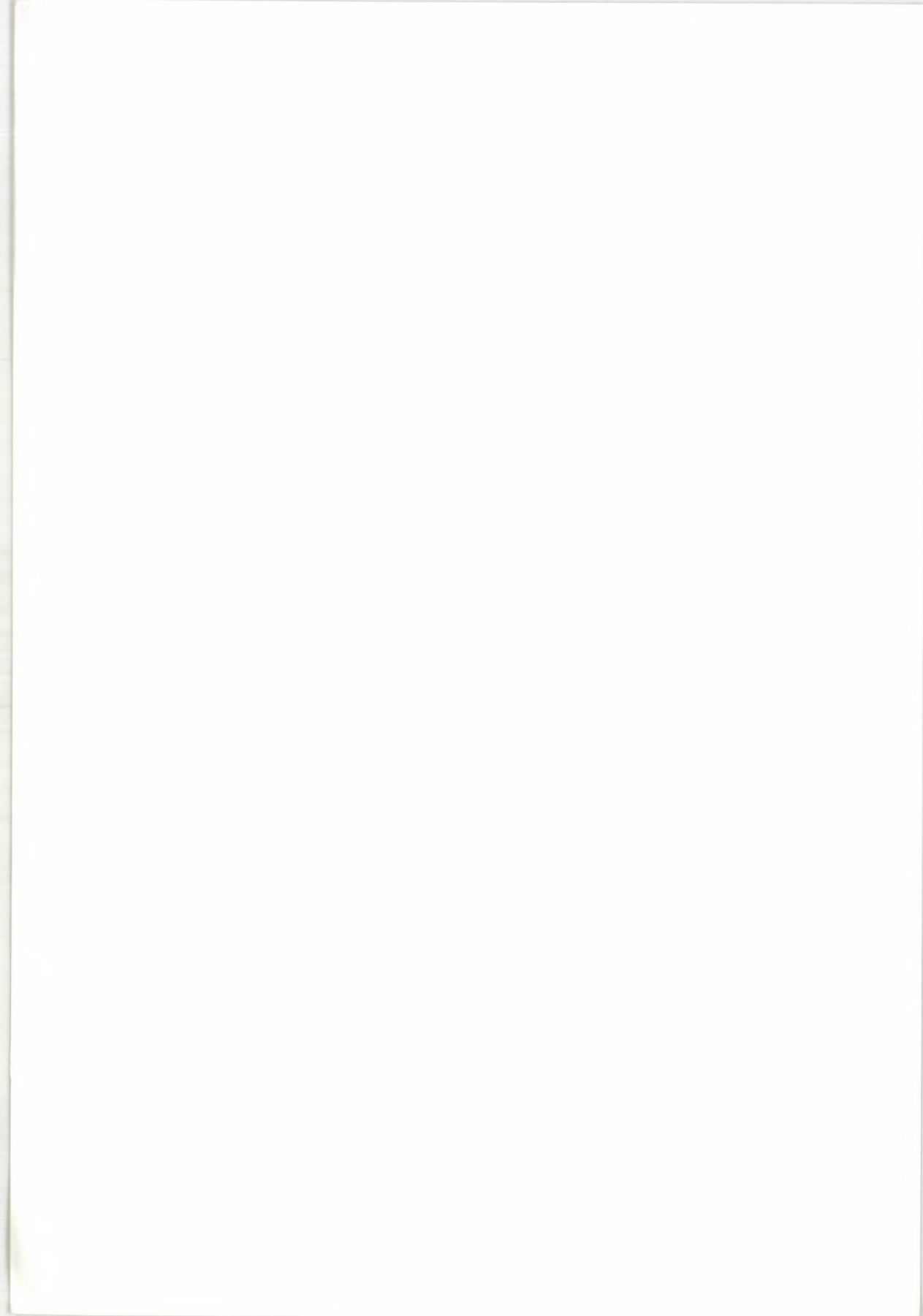
Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója

Műszaki szerkesztő: Zacsik Annamária

A kézirat nyomdába érkezett: 1977. IV. 21. — Terjedelem: 8,05 (A/5) ív 64 ábra

77.4443 Akadémiai Nyomda, Budapest — Felelős vezető: Bernát György



Die *Acta Microbiologica* veröffentlichen Abhandlungen aus dem Bereiche der Mikrobiologie in deutscher, englischer, französischer und russischer Sprache.

Die *Acta Microbiologica* erscheinen in Heften wechselnden Umfangs. Mehrere Hefte bilden einen Band.

Die zur Veröffentlichung bestimmten Manuskripte sind an folgende Adresse zu senden:
Acta Microbiologica, Staatliches Institut für Hygiene, H-1966 Budapest, P.O.B. 64, Ungarn

An die gleiche Anschrift ist auch jede für die Redaktion bestimmte Korrespondenz zu richten. Abonnementspreis pro Band: \$ 32.00.

Bestellbar bei dem Außenhandels-Unternehmen «Kultúra» (H-1389 Budapest 62, P.O.B. 149, Bankkonto Nr. 218-10990) oder bei seinen Auslandsvertretungen und Kommissionären.

Les *Acta Microbiologica* paraissent en français, allemand, anglais et russe et publient des travaux du domaine de la microbiologie.

Les *Acta Microbiologica* sont publiés sous forme des fascicules qui seront réunis en volumes.

On est prié d'envoyer les manuscrits destinés à la rédaction à l'adresse suivante:
Acta Microbiologica, Institut National d'Hygiène Publique, H-1966 Budapest, P.O.B. 64, Hongrie

Toute correspondance avec la rédaction doit être envoyée à cette même adresse.

Le prix de l'abonnement est de \$ 32.00 par volume.

On peut s'abonner à l'Entreprise pour le Commerce Extérieur «Kultúra» (H-1389 Budapest 62, P.O.B. 149, Compte courant No. 218-10990) ou à l'étranger chez tous les représentants ou dépositaires.

«*Acta Microbiologica*» публикуют трактаты из области микробиологии на русском, немецком, английском и французском языках.

«*Acta Microbiologica*» выходят отдельными выпусками разного объема. Несколько выпусков составляют один том.

Предназначенные для публикации рукописи следует направлять по адресу:
Acta Microbiologica, Институт Здравоохранения, H-1966 Budapest, P.O.B. 64, Венгрия

По этому же адресу направлять всякую корреспонденцию для редакции.

Подписания цена — \$ 32.00 за том.

Заказы принимает предприятие по внешней торговле «Kultúra» (H-1389 Budapest 62, P.O.B. 149, Текущий счет № 218-10990), или его заграничные представительства и уполномоченные.

Reviews of the Hungarian Academy of Sciences are obtainable
at the following addresses:

AUSTRALIA

C.B.D. LIBRARY AND SUBSCRIPTION SERVICE,
Box 4886, G.P.O., Sydney N.S.W. 2001
COSMOS BOOKSHOP, 145 Ackland Street, St.
Kilda (Melbourne), Victoria 3182

AUSTRIA

GLOBUS, Höchstädtplatz 3, 1200 Wien XX

BELGIUM

OFFICE INTERNATIONAL DE LIBRAIRIE, 30
Avenue Marnix, 1050 Bruxelles
LIBRAIRIE DU MONDE ENTIER, 162 Rue du
Midi, 1000 Bruxelles

BULGARIA

HEMUS, Bulvar Ruszki 6, Sofia

CANADA

PANNONIA BOOKS, P.O.Box 1017, Postal Sta-
tion "B", Toronto, Ontario M5T 2T8

CHINA

CNPICOR, Periodical Department, P.O.Box 50,
Peking

CZECHOSLOVAKIA

MAD'ARSKÁ KULTURA, Národní třída 22,
115 66 Praha
PNS DOVOZ TISKU, Vinohradská 46, Praha 2
PNS DOVOZ TLACE, Bratislava 2

DENMARK

EJNAR MUNKSGAARD, Norregade 6, 1165
Copenhagen

FINLAND

AKATEMINEN KIRJAKAUPPA, P.O.Box 128,
SF-00101 Helsinki 10

FRANCE

EUROPÉRIODIQUES S.A., 31 Avenue de Ver-
sailles, 78170 La Celle St.-Cloud
LIBRAIRIE LAVOISIER, 11 rue Lavoisier, 75008
Paris
OFFICE INTERNATIONAL DE DOCUMENTA-
TION ET LIBRAIRIE, 48 rue Gay-Lussac, 75240
Paris Cedex 05

GERMAN DEMOCRATIC REPUBLIC

HAUS DER UNGARISCHEN KULTUR, Karl-
Liebknecht-Strasse 9, DDR-102 Berlin
DEUTSCHE POST, ZEITUNGSVERTRIEBSAMT,
Strasse der Pariser Kommune 3-4, DDR-104 Berlin
GERMAN FEDERAL REPUBLIC
KUNST UND WISSEN, ERICH BIEBER, Postfach
46, 7000 Stuttgart 1

GREAT BRITAIN

BLACKWELL'S PERIODICALS DIVISION, Hythe
Bridge Street, Oxford OX1 2ET
BUMPUS, HALDANE AND MAXWELL LTD.,
Cowper Works, Olney, Bucks MK46 4BN
COLLET'S HOLDINGS LTD., Denington Estate,
Wellingborough, Northants NN8 2QT
WM. DAWSON AND SONS LTD., Cannon House,
Folkestone, Kent CT19 5EE
H. K. LEWIS AND CO., 136 Gower Street, London
WC1E 6BS

GREECE

KOSTARAKIS BROTHERS, International Book-
sellers, 2 Hippokratous Street, Athens-143

HOLLAND

MEULENHOF-BRUNA B.V., Beulingstraat 2,
Amsterdam
MARTINUS NIJHOFF B.V., Lange Voorhout
9-11, Den Haag

SWETS SUBSCRIPTION SERVICE, 347b Heere-
weg, Lisse

INDIA

ALLIED PUBLISHING PRIVATE LTD., 13/14
Asaf Ali Road, New Delhi 110001
150 B-6 Mount Road, Madras 600002
INTERNATIONAL BOOK HOUSE PVT. LTD.,
Madame Cama Road, Bombay 400039
THE STATE TRADING CORPORATION OF
INDIA LTD., Books Import Division, Chandralok,
36 Janpath, New Delhi 110001

ITALY

EUGENIO CARLUCCI, P.O.Box 252, 70100 Bari
INTERSCIENTIA, Via Mazzè 28, 10149 Torino
LIBRERIA COMMISSIONARIA SANSONI, Via
Lamarmora 45, 50121 Firenze
SANTO VANASIA, Via M. Macchi 58, 20124
Milano
D. E. A., Via Lima 28, 00198 Roma

JAPAN

KINOKUNIYA BOOK-STORE CO. LTD., 17-7
Shinjuku 3 chome, Shinjuku-ku, Tokyo 160-91
MARUZEN COMPANY LTD., Book Department,
P.O.Box 5050 Tokyo International, Tokyo 100-31
NAUKA LTD. IMPORT DEPARTMENT, 2-30-19
Minami Ikebukuro, Toshima-ku, Tokyo 171

KOREA

CHULPANMUL, Phenian

NORWAY

TANUM-CAMMERMEYER, Karl Johansgatan
41-43, 1000 Oslo

POLAND

WEGIERSKI INSTYTUT KULTURY, Marszał-
kowska 80, Warszawa
CKP I W ul. Towarowa 28 00-958 Warsaw

ROMANIA

D. E. P. București
RÖMLIBRI, Str. Biserica Amzei 7, București

SOVIET UNION

SOJUZPETCHATJ — IMPORT, Moscow
and the post offices in each town
MEZHDUNARODNAYA KNIGA, Moscow G-200

SPAIN

DIAZ DE SANTOS, Lagasca 95, Madrid 6

SWEDEN

ALMQVIST AND WIKSELL, Gamla Brogatan 26,
101 20 Stockholm
GUMPERTS UNIVERSITETSBOKHANDEL AB,
Box 346, 401 25 Göteborg 1

SWITZERLAND

KARGER LIBRI AG, Petersgraben 31, 4011 Base

USA

EBSCO SUBSCRIPTION SERVICES, P.O.Box
1943, Birmingham, Alabama 35201
F. W. FAXON COMPANY, INC., 15 Southwest
Park, Westwood, Mass. 02090
THE MOORE-COTTELL SUBSCRIPTION
AGENCIES, North Cohocton, N. Y. 14868
READ-MORE PUBLICATIONS, INC., 140 Cedar
Street, New York, N. Y. 10006
STECHELT-MACMILLAN, INC., 7250 Westfield
Avenue, Pensacola N. J. 08110

VIETNAM

XUNHASABA, 32, Hai Ba Trung, Hanoi

YUGOSLAVIA

JUGOSLAVENSKA KNJIGA, Terazije 27, Beograd
FORUM, Vojvode Mišića 1, 21000 Novi Sad

ACTA MICROBIOLOGICA

ACADEMIAE SCIENTIARUM
HUNGARICAE

ADIUVANTIBUS

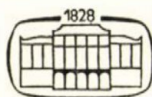
L. ALFÖLDI, I. BÉLÁDI, I. DÖMÖK, E. FARKAS, G. FARKAS,
L. FERENCZY, I. FÖLDES, I. HORVÁTH, I. JOÓ, I. KÉTYI,
L. KESZTYŰS, B. LÁNYI, I. NÁSZ, I. M. SZABÓ, L. VÁCZI,
J. WEISZFEILER

REDIGIT

G. IVÁNOVICS

TOMUS XXIV

FASCICULUS 4



AKADÉMIAI KIADÓ, BUDAPEST

1977

AMAH 5 24(4) 263-345 1977

Acta microbiol. Acad. Sci. hung.

ACTA MICROBIOLOGICA

A MAGYAR TUDOMÁNYOS AKADÉMIA MIKROBIOLÓGIAI KÖZLEMÉNYEI

KIADÓHIVATAL: 1954 BUDAPEST, ALKOTMÁNY UTCA 21.

A Szerkesztő bizottság elnöke:
IVÁNOVICS GYÖRGY
akadémikus

Főszerkesztő:
LÁNYI BÉLA

Az *Acta Microbiologica* német, angol, francia és orosz nyelven közöl értekezéseket a mikrobiológia tárgyköréből.

Az *Acta Microbiologica* változó terjedelmű füzetekben jelenik meg. Több füzet alkot egy kötetet.

A közlésre szánt kéziratok a következő címre küldendők:

Acta Microbiologica, Országos Közegészségügyi Intézet, 1966 Budapest, Pf. 64.

Ugyanerre a címre küldendő minden szerkesztőségi levelezés.

Megrendelhető a belföld számára az Akadémiai Kiadónál (1363 Budapest Pf. 24 Bankszámla 215-11448), a külföld számára pedig a „Kultúra” Külkereskedelmi Vállalatnál (1389 Budapest 62, Pf. 149, Bankszámla: 218-10990) vagy annak külföldi képviselőinél és bizományosainál.

The *Acta Microbiologica* publish papers on microbiological subjects in English, German, French and Russian.

The *Acta Microbiologica* appear in parts of varying size, making up volumes.

Manuscripts should be addressed to

Acta Microbiologica, National Institute of Hygiene, H-1966 Budapest, P. O. B. 64, Hungary

Correspondence with the editors should be sent to the same address.

The rate of subscription is \$ 32.00 a volume.

Orders may be placed with "Kultúra" Foreign Trade Company (H-1389 Budapest 62, P. O. B. 149, Account No. 218-10990) or with representatives abroad.

THE SITE OF ANTIBIOTIC ACCUMULATION IN STREPTOMYCETES AND PENICILLIUM CHRYSOGENUM*

W. KURYŁOWICZ

National Institute of Hygiene, Warsaw, Poland

(Received November 23, 1976)

Morphologic observations and ultrastructural assays of microorganisms have seemingly been dominated by the advances in molecular biology. The microbial anatomy and function of cellular organelles have been learned in detail. Information is, however, rather scarce as to the ultrastructural changes accompanying cell function, and their interplay. Referring to the results on biosynthesis of antibiotics, their major part being reckoned among the secondary metabolites, an attempt was made to obtain more integrated information of the correlation of events in antibiotic biosynthesis and the possible ultrastructural cell modifications. As models for such observations, some *Streptomyces* species were selected, producing about 70% of the antibiotics known to date, as well as *Penicillium chrysogenum* producing benzyl penicillin. Each of the genera (strains) was tested under identical standardized conditions. Two mutants of each strain were assayed, those producing large, and those producing small amounts of the antibiotic. Observations and tests were performed with the material from submerged cultures in laboratory and industrial fermentations. Specimens were taken every 24 hr during the antibiotic biosynthesis cycle. The mycelium surface was assayed by scanning microscopy and the microbial surface and ultrastructure by electron microscopy. In the period of elevated biosynthesis of the secondary metabolites, the presence of amorphous or crystal-like structures was found on the mycelium surface, in which 30 to 70% of the antibiotic produced was detected. In the same period, observation of cell ultrastructure showed in *Streptomyces* numerous membranaceous structures and in *Penicillium chrysogenum* numerous Golgi cisterns. The latter were found by enzymatic and immunological methods to contain the produced benzyl penicillin. The site of antibiotic biosynthesis in the cell, and its transport from cell to the medium, could be determined in *Streptomyces melanochromogenes* and *Penicillium chrysogenum* as models.

Each cell is composed of a large number of physiologically correlated organelles and compartments. Investigations of the cell substructure can therefore reveal important information about cell activity. We have attempted to localize the site of accumulation of the secondary metabolites in the cell. As a model, some antibiotic producing strains of the genus *Streptomyces* and *Penicillium*, and their non-producing mutants were selected.

The nature and relationship of antibiotic synthesis to other metabolic processes of the producing organisms have been a subject for discussion and speculation ever since the discovery of antibiotics.

It has been suggested, for example, that antibiotics may represent waste or breakdown products of cellular metabolism, or products derived from microbial cell wall constituents, substances evolved in the biological antagonism among various species in a natural environment, etc.

* Presented at the Seventh Congress of the Hungarian Society of Microbiology, Budapest, September 6–9, 1976.

Electron microscopic observations of the surface and the fine structure of the antibiotic producing *Streptomyces* strains were performed to determine the site of antibiotic biosynthesis, accumulation and transport from cell into the medium. As for streptomycetes which produce over 60% of all the antibiotics known to date, an attempt was made to elucidate the mechanism by which these Gram-positive microorganisms protect themselves against their own suicidal secondary metabolite.

Electron microscopic assay of the surface and ultrastructure of the mycelium was performed with 5 antibiotic producing streptomycetes.

Strains producing antibiotics with different chemical structures and physical properties were selected. For investigations, the high- and low yielding mutants of the following strains were used. *Streptomyces vinaceus*, producing viomycin, a polypeptide antibiotic; *Streptomyces melanochromogenes*, producing actinomycin D, a peptide antibiotic; *Streptomyces noursei*, producing nystatin, a tetraene antibiotic; *Streptomyces aureofaciens*, producing tetracycline, and *Streptomyces erythreus*, producing erythromycin, a macrolide antibiotic.

The surface of the mycelium of the investigated actinomycetes, observed with a transmission microscope, was smooth in the early hours of fermentation. Later, on the surface of high-yield strains, substructures secreted or excreted from the cells appeared in increasing numbers. Exocellular antibiotic containing substructures were found on the surface of the mycelium of a high-yielding strain of *S. aureofaciens* and *S. noursei* (Figs 1, 2). The surface of the low-yield strains was smooth (Fig. 3). The results indicated that the substructures on the surface of antibiotic-producing actinomycetes have the privileged ability to accumulate secondary metabolites produced by the strain, and probably play a role in the transport of the antibiotic from the cell into the medium [1]. Similar substructures containing antibiotic have been found by CHERNY *et al.* [2-5].

Ultra-thin sections of cells of the high-yielding actinomycetes displayed an electron dense cell wall, and in older hyphae numerous invaginations of cell membrane into the cytoplasm containing electron dense material (Fig. 4). The membrane-bound areas could be associated with production, accumulation, and release of secondary metabolites from the cell into the medium [6].

In the low-yielding strains, the cell walls were also electron-dense, but few membranous structures of the type of vacuoles were seen near the cell membrane.

In the lack of a proper method allowing to identify the antibiotics in the cells of actinomycetes, further studies were performed on *Penicillium chrysogenum* and the benzyl-penicillin produced by this microorganism.

Highly specific enzymatic and immunologic methods made it possible to detect very low amounts of penicillin and these methods were used for localization of this antibiotic in the cells of *P. chrysogenum* in different stages

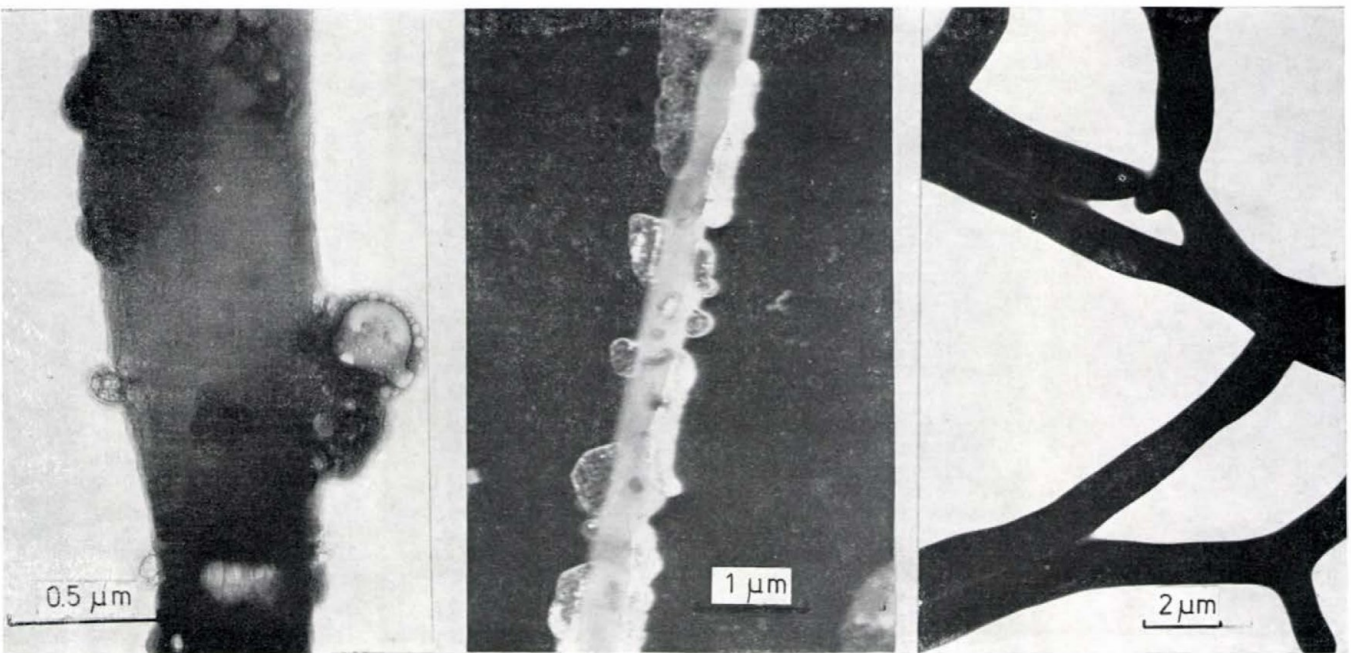


Fig. 1. *S. aureofaciens* var. *tetracyclini*, high-yield strain, 72 hr fermentation. Vesicular substructures on the mycelial surface. $\times 40\,000$

Fig. 2. *S. noursei*, high-yield strain, 74 hr fermentation. Substructures on the mycelial surface. $\times 15\,000$

Fig. 3. *S. aureofaciens* var. *tetracyclini*, low-yield strain, 72 hr fermentation. The mycelium has a smooth surface. $\times 4500$

of biosynthesis. First the ultrastructure of the fungus was studied in submerged cultures during the development cycle [1].

The young hypha was found to consist of three zones: an apical zone, a subapical zone and a zone of vacuolation. In the apical zone, many cytoplasmic vesicles and lomasomes are seen (Fig. 5). The subapical zone is rich in a variety of protoplasmic components: nucleus, mitochondria, endoplasmic reticulum, and ribosomes (Fig. 6). In the third zone the degree of vacuolation increased with the distance from the apex, and a parallel increase in lipid contents was observed (Fig. 7).

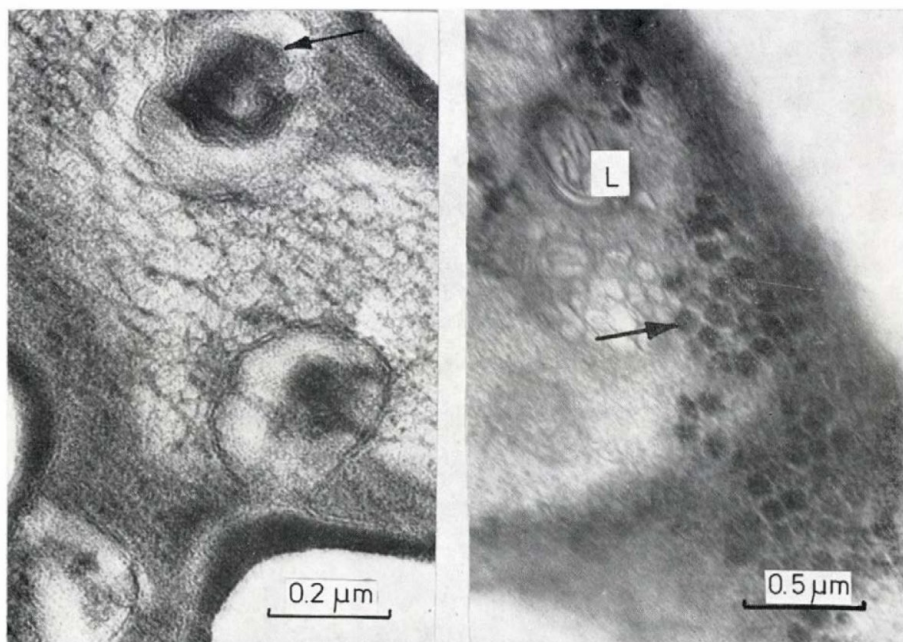


Fig. 4. *S. vinaceous*, high-yield strain, 48 hr fermentation. Invagination of membrane filled with electron-dense material (arrow). $\times 75\ 000$

Fig. 5. *P. chrysogenum*, benzyl-penicillin, high-yield, 24 hr. Near the cell wall of the apical regions a typical lomasome (L), and some characteristic electron-dense wall vesicles are seen (arrow). $\times 30\ 000$

In the cytoplasm of a high-yielding *P. chrysogenum* strain, some multivesicular Golgi bodies were seen (Fig. 8). In the cytoplasm of low-yielding strains these structures were few in number (Fig. 9).

Golgi vesicles occur also in other lower fungi. These vesicles are known to transport some metabolites from the cells into the medium [7, 8].

An analysis of the ultrastructure in relation to antibiotic production, accumulation and secretion in lower fungi has not yet been attempted.

The material for our electron microscopic observations consisted of two *P. chrysogenum* mutants: a high-yielding one, which produced about 10 000 U/ml of benzyl-penicillin, and a low-yielding mutant which produced only about 100 units of benzyl-penicillin per ml.

Two methods of identification of penicillin inside the cell of *Penicillium* were employed. The enzymatic method consists in specific benzyl-penicillin and β -lactamase (penicillinase) binding. Upon staining, the enzyme protein combines with large amounts of heavy metals, thus labelling the sites inside the cell where benzyl-penicillin occurs.

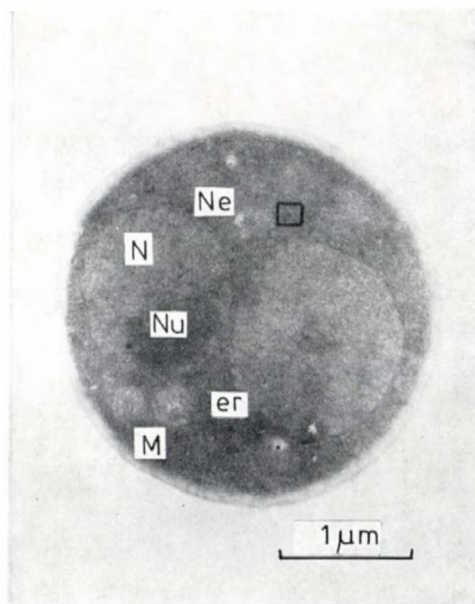


Fig. 6. *P. chrysogenum*, benzyl-penicillin, high-yield, 24 hr. In the nucleus (N) an electron-dense nucleolus (Nu) is often seen. The nucleus is surrounded by a membrane (Ne). In the cytoplasm some mitochondria (M), endoplasmic reticulum (er) and ribosomes (square) are present.
 $\times 17\ 000$

Ultra-thin sections of high-yielding mutant cells treated with β -lactamase displayed many Golgi bodies composed of small vesicles and microtubules, and multivesicular Golgi dictyosomes or single Golgi vesicles filled with electron-dense material (Fig. 10). In preparations treated with inactivated β -lactamase and washed in distilled water these structures were electron-transparent.

The ultra-thin sections of low-yielding mutant cells treated with β -lactamase, showed the electron-transparent membrane bound areas filled with electron-transparent material.

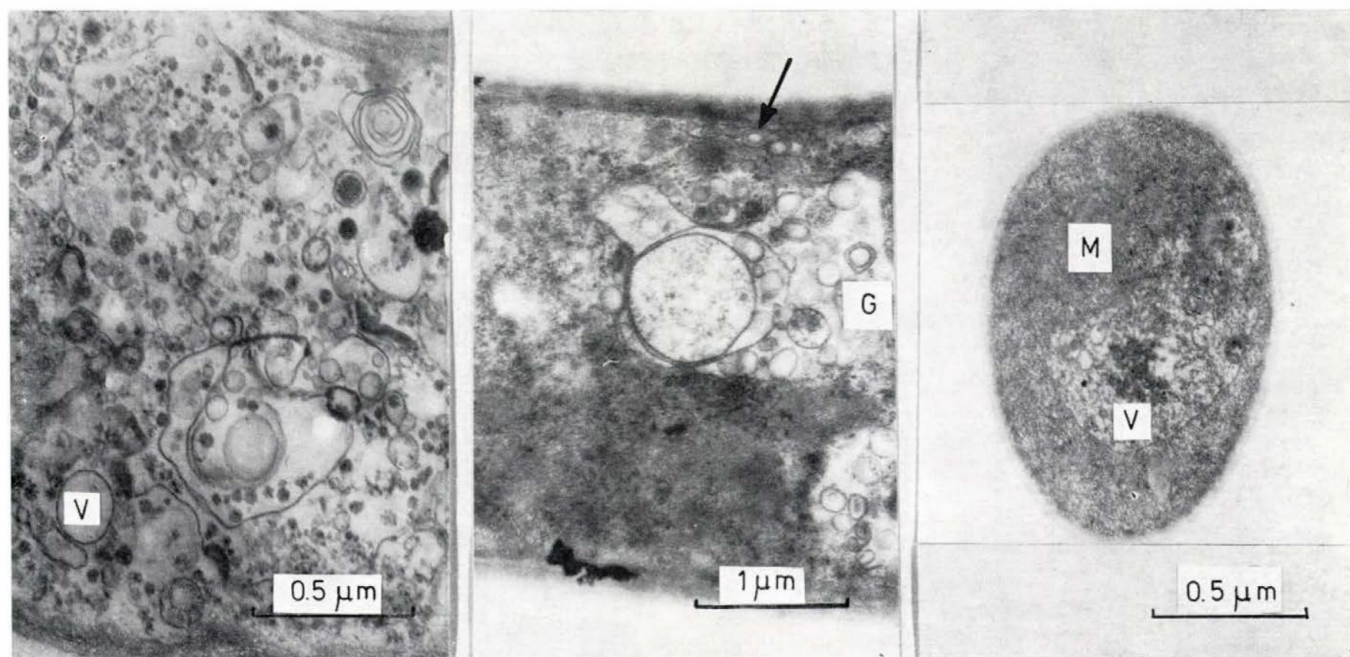


Fig. 7. *P. chrysogenum*, benzyl-penicillin, high-yield, 47 hr. In the cell an extensive membrane system composed of vacuoles (v) is seen. $\times 42\ 000$

Fig. 8. *P. chrysogenum*, benzyl-penicillin, high-yield, 120 hr. Near the cell envelope many Golgi vesicles (G) are present which fuse with the cell membrane (arrow). $\times 20\ 000$

Fig. 9. *P. chrysogenum*, benzyl-penicillin, low-yield, 120 hr. In the cytoplasm no Golgi vesicles are visible. The cytoplasm is densely packed with ribosomes. Mitochondria (M) and vacuoles (v) are also present. $\times 40\ 100$

Thus, the enzymatic method revealed that benzyl-penicillin was accumulated in the Golgi bodies.

The immunologic method of benzyl-penicillin identification inside the cells consists in a specific binding of anti-penicilloyl-poly-L-lysine (PPL) antibodies or β -lactamase antibodies with benzyl-penicillin. The sites of benzyl-

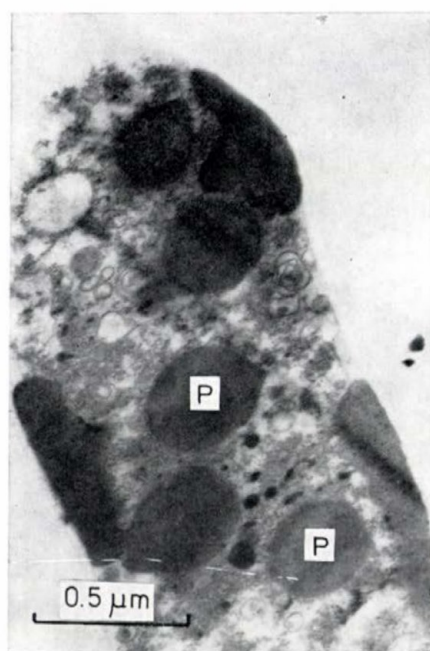


Fig. 10. *P. chrysogenum*, benzyl-penicillin, high-yield, 120 hr. Preparation treated with β -lactamase. In the cell many electron-dense areas, (P); they are probably sites of benzyl-penicillin accumulation in the hypha. $\times 40\,000$

Fig. 11. *P. chrysogenum*, benzyl-penicillin, high-yield, 120 hr. Preparation treated with anti-PPL antibodies. A Golgi body filled with electron-dense material (G). The vesicle is probably the site of benzyl-penicillin accumulation in the hypha. $\times 25\,000$

penicillin accumulation were assayed by ferritin labelled anti-PPL and anti- β -lactamase antibodies.

Ultra-thin sections of the highly-yielding mutant cells treated with specific anti-PPL antibodies showed in the older hyphae Golgi bodies containing numerous vacuoles and single Golgi vesicles filled with electron-dense contents (Fig. 11). Sections treated with inactivated antibodies showed Golgi bodies filled with electron-transparent material. On the contrary, the cells of a high-yield mutant showed single Golgi bodies filled with electron-dense contents packed with electron opaque ferritin deposits (Fig. 12).

In preparations treated with inactivated ferritin labelled antibodies these structures were electron-transparent.

The immunologic method of benzyl-penicillin localization inside the cells with the use of ferritin-labelled anti- β -lactamase antibodies showed in the cells of the high-yielding strain single Golgi vesicles filled with electron-dense contents packed with electron opaque ferritin deposits (Fig. 13).

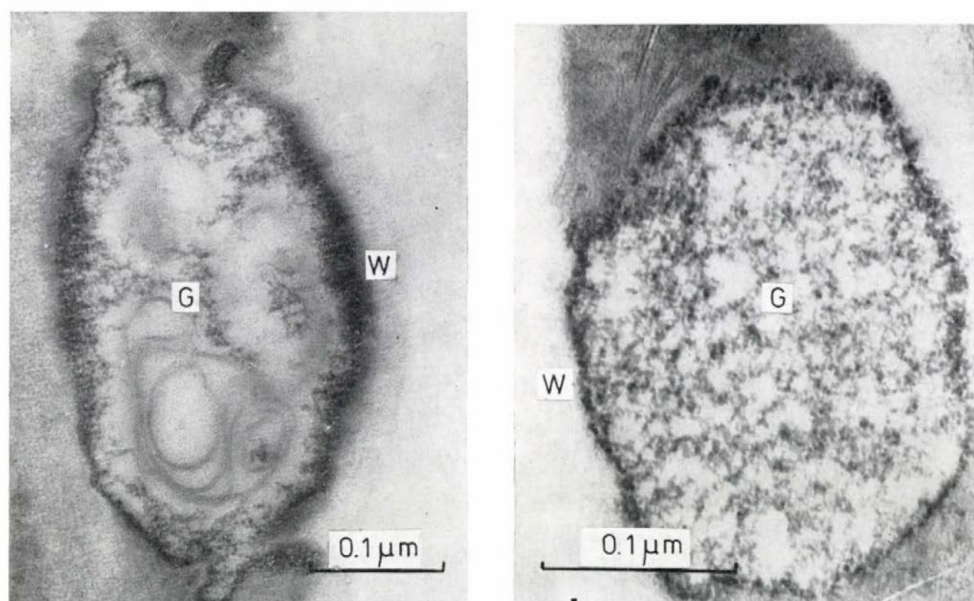


Fig. 12. *P. chrysogenum*, benzyl-penicillin, high-yield, 120 hr. Preparation treated with ferritin-labelled anti-PPL antibodies. Near the cell wall (w) Golgi vesicles filled with electron-dense material packed with electron-opaque ferritin deposits are seen (G). The Golgi vesicles are the probable site of benzyl-penicillin accumulation. $\times 175\ 000$

Fig. 13. *P. chrysogenum*, benzyl-penicillin, high-yield, 120 hr. Preparation treated with β -lactamase and then with ferritin-labelled anti- β -lactamase antibodies. Near the cell wall (w) single Golgi vesicles filled with electron-dense material packed with electron-opaque ferritin deposits (G). $\times 250\ 000$

Thus, all methods showed that the antibiotic was accumulated inside the Golgi bodies. The benzyl-penicillin may then be transported to the cell surface in Golgi vesicles whose membrane — as shown in ultrastructural observations — is capable of fusion with the cell membrane.

According to some data, about 1% of the antibiotic is accumulated intracellularly during the biosynthesis of benzyl-penicillin in *P. chrysogenum*, producing about 7000 units of benzyl-penicillin per ml of the medium [9]. This might suggest a rapid release of benzyl-penicillin from the mycelium into the medium; therefore, the site of the antibiotic accumulation inside the cell may supposedly be also the site of its synthesis.

REFERENCES

1. KURYŁOWICZ, W., MALINOWSKI, K.: *Acta microbiol. pol. Ser. B*, **2** (19) 223 (1970).
2. ЧЕРНИ, Н. Е., ТИХОНЕНКО, А. С., КАЛАФΟΥТСКИЙ, Л. В.: *Макробиология* **41**, 1106 (1972).
3. CHERNY, N. E., TIKHONENKO, A. S., NIKITINA, E. T., KALAKOUTSKII, L. V.: *Cytobios* **5**, 7 (1972). cit. CHERNY, N. E., TIKHONENKO, A. S., KURODA, S., KALAKOUTSKII, L. V.: *Microbios* **10**, 7 (1974).
4. CHERNY, N. E., TIKHONENKO, A. S., KALAKOUTSKII, L. V.: *Microbios* **10**, 65 (1974).
5. CHERNY, N. E., TIKHONENKO, A. S., KURODA, S., KALAKOUTSKII, L. V.: *Microbios* **10**, 7 (1974).
6. KURYŁOWICZ, W., KURZĄTKOWSKI, W., WILLIAMS, S. T., WOŹNICKA, W., PASZKIEWICZ, A.: *Atlas of Ultrastructure of Streptomyces in course of Biosynthesis of Antibiotics*. Polish Medical Publishers, Warsaw 1975.
7. BECKETT, A., HEATH, I. B., McLAUGHLIN, D. J.: *An Atlas of Fungal Ultrastructure*. Longman, London 1974.
8. SMITH, J. E., BERRY, D. R.: *Biochemistry of Fungal Development*. Academic Press, New York 1974.
9. DEMAİN, A. L.: *Antibiot. and Chemother.* **7**, 359 (1957).

Address of the author:

W. KURYŁOWICZ
National Institute of Hygiene
24 Chocimska, Warsaw, Poland

INVESTIGATIONS INTO THE ENCEPHALITOGENIC ACTIVITY OF THE HEMPT VACCINE IN GUINEA PIGS

GEORGETTE NYERGES, ILDIKÓ JASZOVSKY, A. PINTÉR and ZSUZSA HOMPÓ

National Institute of Hygiene, Budapest

(Received November 29, 1976)

Several lots of the Hempt vaccine, a Semple vaccine and the International Reference Preparation of Rabies vaccine were compared with a crude normal sheep brain suspension in respect of their activity to induce experimental allergic encephalitis (EAE) in guinea pigs. Guinea pigs were inoculated intracutaneously with the material to be tested incorporated in Freund's complete adjuvant. Animals were observed for clinical and histopathological signs characteristic of EAE. Using the same inoculation dose, the Hempt vaccine was found much less encephalitogenic than the normal sheep brain and than any of the other vaccines tested.

It has been shown that inoculation of crude adult brain tissue into guinea pigs results in an encephalomyelitis with clinical and histopathological changes resembling those of human neurological accidents following rabies vaccination with adult brain tissue rabies vaccine [1]. Based on these similarities the guinea pig encephalomyelitis (called experimental allergic encephalitis: EAE) has been accepted as a model for studying the postvaccination neurological complications, and the EAE induction method has been recommended to test rabies vaccines for neuromuscular factors [2]. The EAE induction method consists of observing guinea pigs for clinical and/or histopathological changes characteristic of EAE after inoculation with the vaccine incorporated in Freund's complete adjuvant.

Crude adult brain tissue rabies vaccines (Semple-type) were found highly encephalitogenic, while those prepared from the supposedly myelin-free brain of suckling animals proved non-encephalitogenic in the EAE test [3, 4]. Like Semple vaccine, Hempt vaccine is an adult brain tissue rabies vaccine which differs from the former in that its substrate is a sheep brain tissue modified by ether extraction. The Hempt vaccine is still in use in many countries of Central Europe including Hungary; its encephalitogenic activity was tested by FINGER [5] and KUWERT [6]. Their methods for inducing EAE in experimental animals differed much from the generally applied single-inoculation guinea pig test. FINGER inoculated guinea pigs on two occasions with altogether 250 mg vaccine tissue; he found the vaccine almost free of encephalitogenic activity. KUWERT observed no signs of EAE in rabbits inoculated with Hempt vaccine.

The present study deals with the EAE-activity of various lots of the Hempt vaccine as observed in the single-inoculation guinea pig test. Besides, the EAE-activity of the Hempt vaccine was compared with that of other brain tissue vaccines and of a normal sheep brain tissue.

Materials and methods

Vaccines. 1. Hempt vaccines. Seven lots of fluid vaccine (lots 1 - 7) were produced by the National Institute of Hygiene, Budapest, between 1974 and 1976. Two other lots (I and II) were received from abroad. Lot I was a fluid and lot II was a lyophilized preparation. 2. An UV-inactivated vaccine, the 2nd International Reference Preparation for Rabies Vaccine (lyophilized). 3. A phenol-inactivated (Semple-type) vaccine, laboratory reference preparation (lyophilized).

Hempt vaccine lot I and the Semple-type vaccine were kindly supplied by Prof. F. OBERDOERSTER (Staatliches Institut für Serum und Impfstoffprüfung, Berlin), Hempt vaccine lot II by Dr. F. STASTNY (Institute of Sera and Vaccines, Praha).

Normal sheep brain. A suspension prepared from the brain of healthy adult sheep (lyophilized).

Adjuvant. Complete Freund's adjuvant (CFA, Difco).

Animals. Hartley guinea pigs weighing 300-500 g.

Test for encephalitogenic activity. Suspensions were prepared to contain the amount of the brain tissue to be inoculated in 0.3 ml. from the sediment of the centrifuged fluid vaccines and from that of the reconstituted lyophilized preparations. Before inoculation the suspension was thoroughly mixed with the same volume of CFA to give a satisfactory emulsion. (The drops when shed on the surface of distilled water did not spread over it). Guinea pigs were inoculated by 3 intracutaneous injections of 0.2 ml each in 3 areas of the shaved skin of the back. Each preparation was tested on 5 animals. The guinea pigs were kept under observation for 30 days. Paralysis of at least one leg developing between the 10th and 16th postinoculation day was accepted as the clinical criterion for EAE. The severity of EAE was graded from + to +++ according to the degree and extension of paralysis. The animals were necropsied when moribund or, if healthy or not seriously ill, at the end of the observation period. Brains and the medullas were embedded in paraffin and sections were stained according to Nissl's method and examined for changes characteristic of EAE. The severity of EAE was graded from + to +++ according to the extension of the perivascular lymphocytic infiltration (Figs 1-3). In the first part of the study, animals were examined for histopathological changes regardless of the clinical signs. After having found that clinical EAE was invariably accompanied by typical histological changes, paralysed animals were examined histologically only occasionally. The majority of the clinically healthy animals was observed for histopathological changes throughout the study.

Results

1. *Incidence of EAE in guinea pigs inoculated with rabies vaccines.* Nine lots of Hempt vaccine, the UV-inactivated International Reference Preparation and the phenolized (Semple-type) reference vaccine were examined for encephalitogenic activity in the course of 7 experiments. When not indicated otherwise, 70 mg native brain were inoculated from the sediment of the centrifuged fluid Hempt vaccines (lots 1 through 7 and I) and from that of the reconstituted lyophilized vaccine (lot II) and the Semple-type vaccine. From the lyophilized normal sheep brain and from the International Reference Preparation 14 mg lyophilized brain substance were inoculated, since we observed that native

Table I

Incidence of EAE in guinea pigs given rabies vaccine or normal sheep brain in CFA

Experiment	Material	Dose (mg native brain)	No. of animals		No. of clinically negative animals with histological changes of EAE*
			with	without	
			clinical signs of EAE		
A	Int. Reference	70	4	0	
B	Simple	70	3	2	1/2
B	Hempt lot 1	70	0	5	0/3
C	Hempt lot 1	70	0	5	2/5
D	Hempt lot 2	45	1	4	4/4
E	Hempt lot 2	22,5	0	5	1/3
		5.6	0	5	0/3
		1.4	0	5	1/4
A	Hempt lot 2	70	0	5	3/5
C	Hempt lot 2	70	0	5	0/5
B	Hempt lot 3	70	0	5	2/3
C	Hempt lot 3	70	0	5	2/5
C	Hempt lot 4	70	2	3	0/2
F	Hempt lot 5	70	0	5	0/5
G	Hempt lot 6	70	0	5	1/5
G	Hempt lot 7	70	0	5	0/5
F	Hempt lot I	70	1	4	0/4
G	Hempt lot II	70	0	5	0/3
A	Normal sheep brain	70	5	0	
B	Normal sheep brain	70	3	2	2/2
C	Normal sheep brain	70	3	2	1/2
D	Normal sheep brain	70	3	2	n.t.**
E	Normal sheep brain	70	5	0	
F	Normal sheep brain	70	5	0	
G	Normal sheep brain	70	3	2	1/2

* positive/tested

** not tested

sheep brain lost 80% of its original weight during lyophilization. The normal sheep brain preparation was included in every experiment. In Table I results are grouped according to the type of the vaccines tested. The first column shows the 7 experiments indicated by letters A to G so that the tests carried out at the same time can be compared. Doses are expressed in mg native brain.

In the control groups every animal developed clinical symptoms of EAE in 3 experimental series, and 3 out of 5 animals in 4 experimental series.

Out of 8 symptom-free animals 6 were examined histologically; EAE was found in 4 cases. Data of the guinea pigs inoculated with the International Reference Preparation and of those receiving the Semple-type vaccine were almost similar to those of the controls. Hempt vaccine caused clinical EAE very rarely. One animal had characteristic clinical symptoms in response to the inoculation of 45 mg brain tissue and the inoculation of 70 mg



Fig. 1. Mild mononuclear cell infiltration involving meninges of the spinal cord. Klüver-Barrera stain. $\times 25$

was followed by paralysis in 3 out of 60 cases. Sixty-four clinically healthy animals were examined histologically, 15 of them proved positive for EAE. In a few cases the EAE test was repeated. Lot 3 was tested in both B and C experiments, the results were almost identical *i. e.* no clinical EAE was observed while the histological examination indicated EAE in some cases. In contrast, 1 guinea pig became paralysed and each of the remaining 4 animals exhibited typical histological changes after the inoculation of 45 mg brain tissue from lot 2 (experiment D). The same vaccine, however, induced only histological changes in experiment A, and proved entirely safe in experiment C.



Fig. 2. Extensive moderate lymphocytic infiltration around intracerebral capillaries in guinea pig brain. Nissl stain. $\times 40$



Fig. 3. Extensive round cell and polymorphonuclear cell infiltration of vessel walls and of adjacent brain. Nissl stain. $\times 40$

Animals inoculated with 70 mg normal sheep brain developed much more serious EAE than did those inoculated with the same amount of the Hempt vaccine. Thus, 77% (27/35) of the control animals exhibited clinical EAE with grave paralysis in 59% of the cases (16/27). In contrast, only 5% (3/60) of the vaccine-treated animals manifested clinical EAE and no grave

Table II

Incidence of clinical signs and histologic changes of EAE in guinea pigs given different doses of normal sheep brain in CFA

Experiment	Dose (mg native brain)	No. of animals		No. of clinically negative animals with histologic lesions*
		with	without	
		clinical signs of EAE		
1	70.0	5	0	not tested
	23.5	5	0	
	8.0	5	0	
	2.5	2	3	
	1.0	4	0	
2	70.0	5	0	0/2 2/5 1/4
	0.3	2	3	
	0.1	0	5	
	0.035	0	5	

* positive/tested

paralysis occurred. Although EAE could be proved histologically in 10 of 50 clinically negative vaccine-treated animals, the changes were moderate in the majority (9/10) of cases (experiments A, B, C, F, G).

2. *Minimal encephalitogenic dose of normal sheep brain.* Two experiments were performed to determine the minimal encephalitogenic dose of the control brain substance. The data are presented in Table II. Doses are expressed in mg native brain. Two experiments were carried out. In the first trial no histological examination was done, but as little as 1 mg proved encephalitogenic. Results of the second experiment showed that 0.3 mg induced clinical EAE, and 1 out of 5 animals inoculated with as little as 0.035 mg exhibited histopathological changes characteristic of EAE.

Discussion

Both clinical and histological examinations proved that Hempt vaccine had a much lower encephalitogenic activity than the normal, *i. e.* the crude sheep brain. The minimal paralyzing dose of the crude sheep brain ranged between 0.1 to 0.3 mg and the minimal dose causing histological changes of EAE was found to be about 0.035 mg. For the ether-treated brain tissue in

the vaccine, the minimal paralyzing dose was estimated at 22.5 to 45 mg and the minimal encephalitogenic dose at 5.6 to 22.5 mg (Table I, experiments D and E). Although the data are few for establishing a definite quantitative relation, they suggest that the encephalitogenic activity of the normal sheep brain must be about 2 log higher than that of the brain tissue in the Hempt vaccine. The great majority of the control animals developed clinical EAE with grave paralysis in most cases. In contrast, animals inoculated with the same amount of the vaccine tissue exhibited clinical EAE exceptionally, and no grave paralysis was noted. The marked differences between the crude and the ether-treated brain tissue leave little doubt that Hempt vaccine can be regarded as a purified adult brain tissue vaccine.

The encephalitogenic activity of the Hempt vaccine proved considerably lower than that of the UV-inactivated vaccine or of the Semple-type vaccine. No comparisons were made with suckling brain vaccines, but data of GISPEN *et al.* [4] suggest that the Hempt vaccine is in all likelihood more encephalitogenic than is the suckling rabbit brain vaccine. Comparison of our results to GISPEN's data seems to be justified because both the doses and the method of the EAE test were similar in both investigations.

The lots of the Hempt vaccine examined differed somewhat in encephalitogenicity (Table I); *e. g.* lot 4 was found more reactive than all the others. As our results were not always reproducible, further observations are needed to investigate whether real differences occur. Incongruent results were encountered only with the vaccines but this was not the case with the control preparation. This may have been due to the fact that 70 mg may correspond to the 50% encephalitogenic dose of the ether-treated brain tissue, while it is more than that of the crude brain tissue.

The response of the animals to the brain tissue clearly depends on the dose inoculated, the route of inoculation, and the sensitivity of the guinea pig strain used [1, 7]. Therefore, the results of any EAE test carried out with arbitrarily chosen doses is difficult to evaluate in the human relation. Accordingly, the significance of the possible differences in encephalitogenic activity between lots of the Hempt vaccine also remains to be established. Altogether 3000 subjects were subjected to postexposure prophylaxis with lots 1 through 7. No neurological complications were reported, but the number of observations is too small for any conclusion to be drawn. According to FORNOSI [8, 9] data of the last 20 years indicate that the neurological complication rate for the Hempt vaccine can be estimated at 1/5170 in Hungary. As in many other countries, we have also seen marked yearly fluctuations in the incidence of post-vaccination complications. Accordingly, in 1967, 4 complications occurred among 2069 persons subjected to postexposure prophylaxis, while no complication was observed among 3174 persons subjected to similar treatment in 1968. Routine testing for encephalitogenic activity of the vaccine seems to be the

only means to find out how complication rates are reflected in the laboratory results, *i. e.* whether the EAE test as applied by us is a useful method for predicting the reactogenicity of the Hempt vaccine.

REFERENCES

1. PATERSON, PH. Y.: *Advanc. Immunol.* **5**, 131 (1966).
2. GISPEN, R.: In KAPLAN, M. M., KOPROWSKI, H. (eds): *Laboratory Techniques in Rabies*. WHO, Geneva 1973. pp. 295—298.
3. SVET-MOLDAVSKIY, G. JA., ANDJAPARIDZE, O. G., UNANOV, S. S., KARAKAJUMCAN, M. K., SVET-MOLDAVSKAJA, I. A., MUCNIK, L. S., HIENINSON, M. A., RAVKINA, L. I., MTVARE-LIDZE, A. A., VOLKOVA, O. F., KRIEGSHABER, M. R., KALINKINA, A. G., SALITA, T. V., KLIMOVICKAJA, V. I., BONDALETOVA, I. N., ROJHEL', V. M., KISELEVA, I. S., LEVCENKO, E. N., MARENNIKOVA, S. S., LEONIDOVA, S. I.: *WHO Techn. Rep. Ser.* **32**, 47 (1965).
4. GISPEN, R., SCHMITTMANN, G. J. P., SAATHOF, B.: *Arch. ges. Virusforsch.* **15**, 366 (1964/65).
5. FINGER, H.: *Z. Immun. Forsch.* **121**, 291 (1961).
6. KUWERT, E.: *Arch. Hyg. Bakt.* **151**, 131 (1967).
7. OLITSKY, P. K., LEE, J. M.: *J. Immunol.* **71**, 419 (1953).
8. FORNOSI, F.: Personal communication (1976).
9. FORNOSI, F.: *Magy. Pediat.* **2**, 54 (1971).

Address of the authors:

GEORGETTE NYERGES, ILDIKÓ JASZOVSKY, ALÁN PINTÉR, ZSUZA HOMPÓ
National Institute of Hygiene
H-1966 Budapest, P. O. B. 64, Hungary

VITAL FLUORESCENCE MICROSCOPY OF LYSOSOMES IN CULTURED MOUSE PERITONEAL MACROPHAGES DURING THEIR INTERACTIONS WITH MICROORGANISMS AND ACTIVE SUBSTANCES

I. VITAL STAINING AND FLUORESCENCE MICROSCOPY OF MACROPHAGES

T. N. KHAYKIN, I. S. FREIDLIN, I. YA. SAKHAROVA and V. A. IOFFE

*Institute of Experimental Medicine of the U. S. S. R. Academy of Medical Sciences and First
Medical Institute I. P. Pavlov, Leningrad, U. S. S. R.*

(Received December 5, 1976)

Vital staining of macrophage lysosomes under normal conditions is described. Quinacrine (2 $\mu\text{g/ml}$) causes selective fluorescence of lysosomes (red granules) without nuclear staining. Green fluorescence of the nuclei shows some damage of the cultured cells. Fluorescence intensity of individual red granules in the macrophage as well as granule fluorescence intensity of the entire cell, show some heterogeneity of macrophage populations depending on lysosome number in different cells, predominant size of lysosomes, and intensity of their fluorescence. A comparison of the fluorescent pictures and Pappenheim staining of the same cells showed the identity of red granules (lysosomes) and azurophilic granules in cultured mouse peritoneal macrophages.

Observation of lysosomes in living cells cultured *in vitro* provides fresh opportunities of studying the functional states of these cell. Selective staining with fluorescent substances should receive particular attention among the techniques of vital study of lysosomes. These substances generally include aminoacridines, *e. g.* acridine orange and related derivatives, monomers which give a green, and their polymers a red fluorescence [1]. Aminoacridines show a highly pronounced affinity for lysosomes. As their concentration in lysosomes increases, polymers are formed and therefore the lysosomes acquire the form of granules showing red fluorescence [2]. At lower concentrations of fluorochrome, the fluorescence of lysosomes is only seen. The fact that it is lysosomes that appear as red fluorescent granules has been proved by both cytochemical [2] and immunological [3] methods.

Staining of cultured cell lysosomes with fluorescent dyes was applied in the studies on responses to damaging agents and viral invasion [4–6]. The cells stained with aminoacridine fluorochromes may be employed in the studies on interactions of microorganism and lysosomes in the living cell.

COHN and BENSON made a detailed study of lysosomes within macrophages [9–11]. They used neutral red to detect lysosomes and cells by light microscopy. The fluorescence technique, which is much more sensitive, has hardly been used in the investigations of macrophage lysosomes.

The present paper discusses the application of vital fluorescence staining of macrophages and their lysosomes in normal cell cultures and during their

interactions with various living and non-living agents. It also describes the employed model — a macrophage culture containing lysosomes stained with fluorochromes.

Materials and methods

Macrophage culture. Macrophages were obtained from the peritoneal cavity of unstimulated albino mice (10^6 cells/ml medium) and maintained as monolayers on glass coverslips in Leighton tubes containing 1 ml of medium 199 (15% calf serum, 100 mg/ml streptomycin, 1000 U/ml penicillin and 5 U/ml heparin). Two hours after the commencement of incubation, the medium was removed together with unattached cells and incubation was continued in the same medium without heparin. Coverslips containing a cell layer were mounted on slides.

Dyes. Macrophage cultures were stained with methylene-azure-eosin after Pappenheim; lipids were stained with oil red. Acid phosphatase was assayed after BARKA and ANDERSON [12] using hexazonium pararosaniline as coupler.

Fluorescence staining. Quinacrine was used, because at proper concentrations it causes the fluorescence of lysosomes only [1]. The optimum dose proved to be $2 \mu\text{g/ml}$ medium. Macrophage cultures were incubated with quinacrine for 1 hr; subsequently, they were washed three times and maintained in fresh medium without quinacrine.

Microscopy. For fluorescence and phase-contrast microscopy a ML-2 microscope (LOMO, Leningrad) was used, equipped with glass ocular filters for selective filtration of green and red fluorescence. Black-and-white photographs with and without colour selection were taken on RF-3 fluorographic film. Reversible Orwochrom film was used for colour photography. For more details concerning the technique, see [13].

Microfluorimetry. The fluorescence intensity of separate red granules in the cell was measured photographically [14].

The total fluorescence intensity of granules in a cell was determined by a two-ray impulse microfluorometer [15]. In this case, fluorescence was induced by blue-violet light and a light-orange barrier filter was used. Fluorimetry was carried out on 48-hr macrophage cultures because these cells are homogenous in size at this stage. Sampling of cells, their mounting in an optical probe and focussing were performed in visible light with phase contrast to avoid fluorescence fading before measurements. The results of microfluorometric studies were expressed in agreed units.

Results

Lysosomes in the form of red fluorescent granules (colour plate, Fig. 1) fill the endoplasm in the quinacrine-treated macrophages. There are no fluorescent granules in the ectoplasm which constitutes the undulating membranes. Therefore, when fluorescence techniques are used, cells seem smaller in size, than in phase-contrast microscopy (Fig. 2). Red granules show a uniform distribution throughout the cell or they form morula-like aggregates. Granules, if any, generally surround vacuoles (Fig. 3), but sometimes, vacuoles contain red fluorescent material, too. Some cells may incorporate a material showing green fluorescence. Pappenheim staining reveals that such inclusions are the ingested nuclei of dead cells (Fig. 4).

In phase-contrast microscopy, red granules look like dense inclusions. In native preparations, their appearance is similar to that observed by COHN and BENSON [9] after osmium fixation. As a rule, red granules gave a positive acid phosphatase reaction. After the cytochemical reaction the granules seemed,

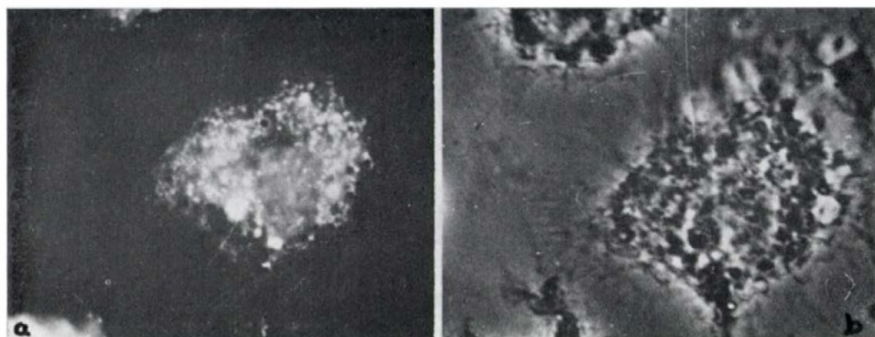


Fig. 2. Fluorescence (a) and phase-contrast (b) images of the same living macrophage. Ectoplasmic areas of the cell (arrows) are distinguished by phase-contrast only, lysosomes are located in the endoplasm. $\times 1000$

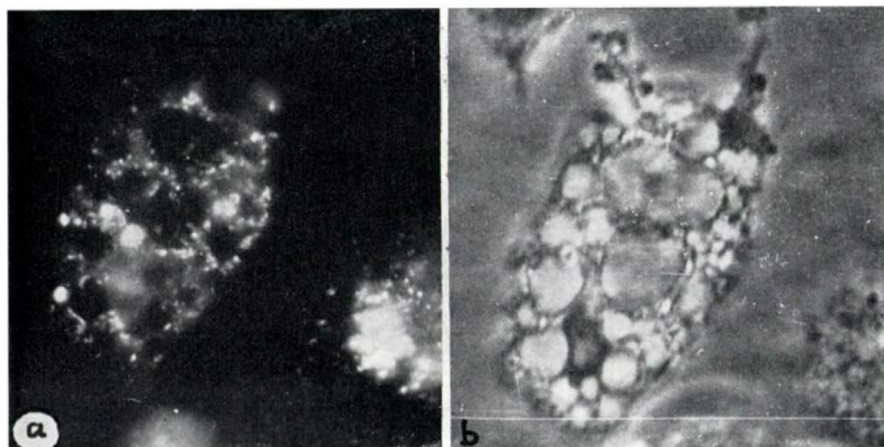


Fig. 3. Fluorescence (a) and phase-contrast (b) images of macrophages containing many pinocytotic vacuoles. Vacuoles are surrounded by lysosomes. $\times 1000$

however, to be less discrete than in vital microscopy. The contents of vacuoles fluorescing in red, gave a very slight acid phosphatase reaction. In some vacuoles the product of the reaction was on the vacuole walls only (Fig. 5); the reaction seems to involve a slight diffusion and loss of enzyme.

Long-term irradiation of the preparation results in a green fluorescence of the nucleus, followed by that of the cytoplasm. This is accompanied by a simultaneous decline in the intensity of the red fluorescence of granules.

Subsequently, the situation changes: though 90–96% of the cells still contain red granules 24 hr after staining, fusion of some granules occurs. The proportion of the granule-containing cells falls to 20–40% of the whole population after 48–82 hr; some cells reveal fewer granules (Fig. 6).

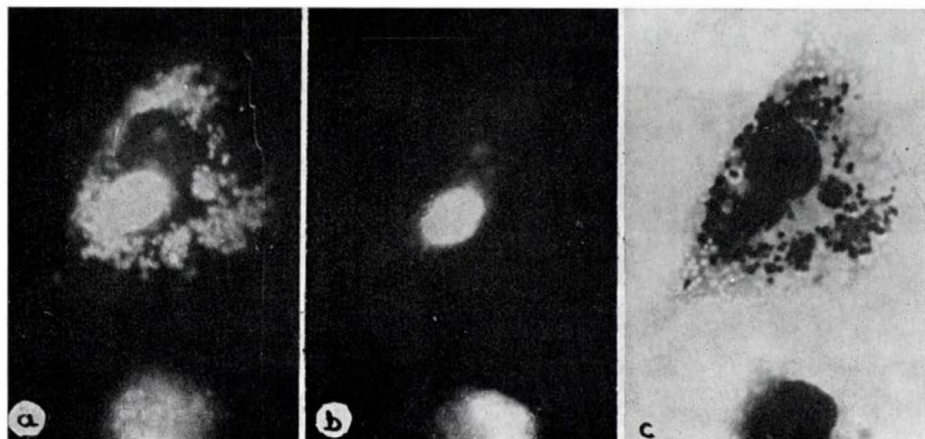


Fig. 4. Macrophage stained with quinacrine, showing red fluorescence of lysosomes and green fluorescence of an ingested dead cell; (a) picture of red and green fluorescence; (b) picture of selective green fluorescence of dead cell; (c) the same cell stained after Pappenheim, showing identity between red fluorescent and azurophilic granules. $\times 1000$

Measurement of total red granule fluorescence in the cell. Preliminary measurements in preparations not treated with fluorescent dyes showed an autofluorescence of the cells and background in the order of 1–2 units; therefore, their contribution to the results of measurement is negligible.

The fluorochrome technique revealed a wide variety of sizes, intensity of fluorescence and numbers of red granules in different cells. It was, however, possible to distinguish 3 groups in the macrophage population on the criterion of total intensity of cell fluorescence (Table I). The ratio of the mean total intensities of cell fluorescence, 10 : 3 : 1 was approximately the same in different experiments. It is clear from Table II that the total intensity of cell fluorescence is a function of two parameters, the content of red granules, and the predomi-

Table I

*Total intensity of macrophage fluorescence with quinacrine labelled lysosomes**

Groups of cells with different red granule content	Mean fluorescence intensity	Mean $\log_{10}$ of single cell fluorescence intensity
High content	1500	3.1 ± 0.25
Medium content	450	2.6 ± 0.26
Low content	150	2.2 ± 0.27

* The results are based on 18 experiments (fluorometry of 421 cells)

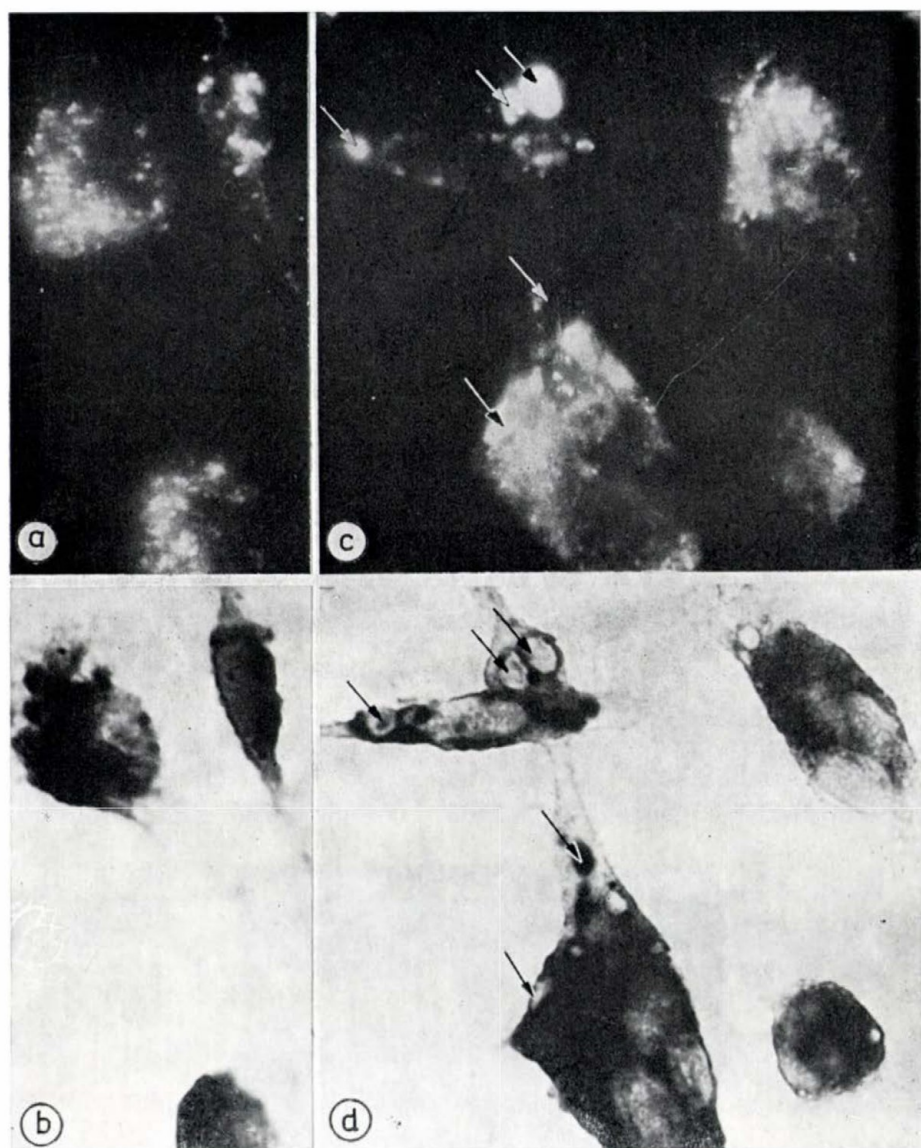


Fig. 5. Cultured macrophages after quinacrine uptake (a, c) and after acid phosphatase reaction (b, d). There is a certain resemblance between the fluorescent and cytochemical images of the same cells but some vacuoles with red fluorescence (arrows) show lacking or weak peripheral enzyme activity. $\times 1200$

nant size of granules. The brightest fluorescence is generally observed in cells containing many large granules and weakest in those containing few small granules. The total fluorescence of a cell is determined less by the fluorescence intensity of individual granules, although it varies within a wide range of mag-



Fig. 6. Cultured macrophages 96 hr after uptake of quinacrine, showing disappearance and/or confluence of red granules

nitude (Fig. 7). There is hardly any correlation between the size and shape of the cells and the total intensity of their fluorescence.

On the basis of such fluorometric data, we were able to assess visually 48 hr macrophage cultures on the basis of the red granule content of the cell. The above three categories of cells, depending on the mean intensity of fluorescence, were established in every population (at least, 100 cells in each preparation), and the parameters shown in Table III were computed for each popula-

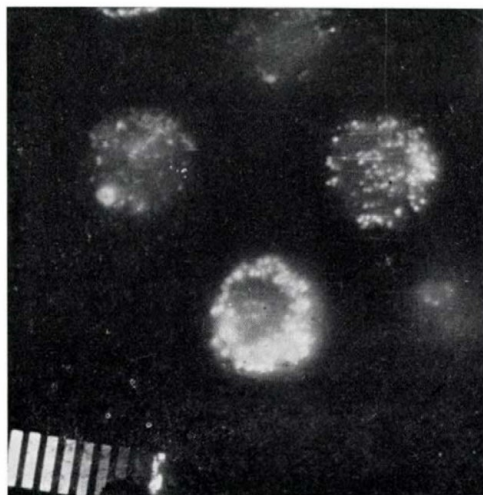


Fig. 7. Photomicrograph of vitally stained macrophages and fluorimetric stepped wedge. Figures (without brackets) denoted fluorescence intensity of some individual red granules and mean fluorescence intensity of granules per cell (in brackets). $\times 1200$

Table II

Macrophage fluorescence intensity depending on the content and predominant size of their lysosomes labelled with quinacrine

Predominant size of red granules in the cells	Red granule content per cell and mean total fluorescence intensity of these cells		
	High	Medium	Low
Large	3.20	2.68	2.59
Medium	3.08	2.57	2.19
Small	2.91	2.44	2.19

Table III

Content of the cells with different total fluorescence intensity in 48-hour-old macrophage culture

Groups of cells with different red granule content	Mean percentage of cell categories per population	
	per cent	range
High content	52.5	43—69
Medium content	34.9	21—48
Low content	12.6	7—19

tion. Table III demonstrates that the cells with a high or medium red granule content constitute nearly 88% of the population. The cells with a high or medium acid phosphatase content in similar 48 hr cultures also constitute 88.8% of the whole population.

Pappenheim staining. Azurophilic granules were invariably revealed *in vitro* in cultured macrophages. Three main types of granules could be distinguished: small, vaguely-outlined, ca. 0.5 μ m wide; medium-size, distinctly-outlined, 1.0—1.5 μ m wide, and large granules, up to 2.0—2.5 μ m wide. Each type of granule showed a peculiar shade of azurophilia. Pappenheim staining after a fluorescence study revealed that granules showing red fluorescence and azurophilic ones were identical. This was particularly apparent in the case of medium and large granules (Fig. 4). Smaller granules were less distinguishable after staining than after fluorochrome treatment (Fig. 8). A particular type of granules was predominant in separate cells. Calculations demonstrated that cultures obtained from the same pool of cells (from 10 and more mice) were characterized by certain correlations between the number of cells containing a particular type of granule in a population. No azurophilic grains could be discerned in many round slightly basophilic cells several hours after

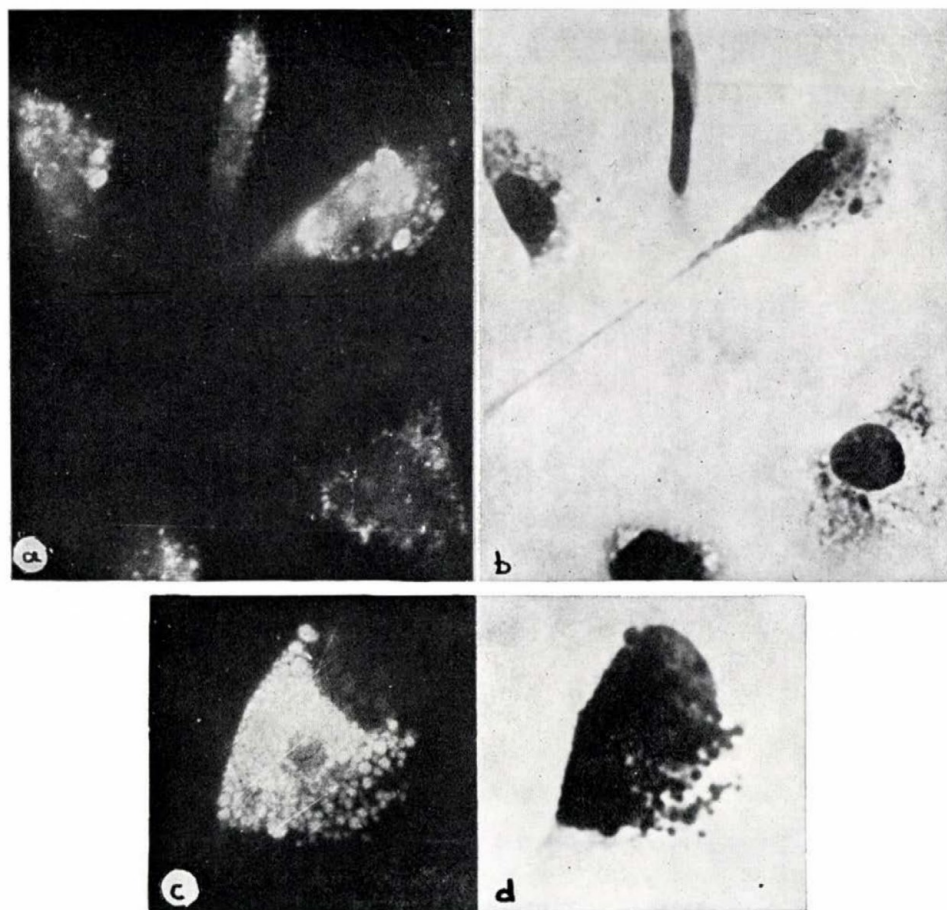


Fig. 8. Cultured macrophages after vital uptake of quinacrine and after Pappenheim staining. There is certain resemblance between the distribution of the small red (a) and azurophilic (b) granules. Large fluorescent (c) and azurophilic granules (d) are identical. $\times 1000$

Table IV

Azurophilic granules in cultured mouse peritoneal macrophages

Cell categories depending on predominant type of granules	Duration of cell cultivation (hours) and percentage of cell categories in populations			
	2h	24h	48h	72h
Granules not distinguishable	70.2 ± 3.7	15.5 ± 2.9	4.5 ± 1.8	2.2 ± 1.1
With small granules	15.3 ± 2.9	34.3 ± 3.9	71.0 ± 3.7	54.2 ± 4.1
With medium granules	14.2 ± 2.7	41.6 ± 4.0	20.5 ± 3.3	40.3 ± 4.0
With large granules	0.17	8.3 ± 2.2	5.2 ± 1.8	3.3 ± 1.4

macrophages had become attached to the glass. Granules became discernible nearly in all cells at later stages, the ratios of the cell types varying slightly after 24, 48 and 72 hr (Table IV).

Discussion

The results obtained in the present study confirm the findings of ROBBINS and MARCUS [16], DINGLE and BARRETT [2] and ISINA and KORN [3] that the phase-dense granules, fluorescing red after vital staining with aminoacridines at low concentrations, are actually lysosomes. This is also supported by acid phosphatase being found in the granules which were revealed by means of fluorochrome treatment.

Since quinacrine selectively stains the lysosomes, it was possible to assess the lysosome content on the basis of the total fluorescence of cells and to interpret the appearance of a green fluorescence of the cell. Such a damaging factor, both in this study and in other investigations [4, 17] was provided by irradiation which caused fluorescence.

Since red fluorescence was sometimes observed in separate vacuoles, apart from phase-dense granules, there may be two explanations: (a) the contents of such vacuoles promoted the delivery of quinacrine-labelled hydrolases from lysosomes to vacuoles, and (b) a redistribution of the fluorochrome had taken place. The former hypothesis is consistent with the concept of DINGLE and BARRETT [2], KOENIG [18] and ZELENIN [19] that aminoacridines are bound to hydrolases contained in lysosomes. This hypothesis is further supported by the presence of traces of acid phosphatase in some vacuoles showing red fluorescence. The above hypothesis on fluorochrome redistribution is at variance with the reports on a high affinity of aminoacridines for lysosomes, which is 10–15 times higher than for other cell organoids. [2].

Variations in the fluorescence pattern at different stages after staining are most likely to represent the process of aminoacridine digestion in lysosomes, as observed by electron microscopy [20]. It is therefore necessary to examine cells immediately after fluorochrome treatment in order to obtain an adequate pattern of lysosome fluorescence.

The present study established the degree of lysosome azurophilia for macrophage cultures. NICHOLS *et al.* [21] maintain that, among mononuclear phagocytes, the lysosomes of the bone marrow and blood monocytes alone are azurophilic, whereas the vesicles formed by the macrophages in body cavities are not. Our experiments showed some lysosomes of macrophages to take up as much azure as those of the monocytes or PMN of blood.

The results showed, further, that macrophage populations are heterogeneous, as far as the lysosome number in different cells, the predominant size of lysosomes and post-quinacrine fluorescence intensity are concerned. Discus-

isons on the functional and morphological heterogeneity of macrophages have a long record [22, 23], but it is not clear whether the macrophage species used in the present study may be correlated with those employed by other authors. Similarly uncertain is the fact whether the above heterogeneity is a manifestation of the different condition of cells or divergence in macrophage maturation.

A comparison of the results of fluorometry and the acid phosphatase reaction showed that the total fluorescence of quinacrine-stained macrophages can be used for the assessment of the cell content of lysosomes and the proportions of cells with different lysosome contents in normal cultures are more or less stable. This suggests the application of methods for the quantitative assessment of the lysosome content in living cells in investigations on various effects of macrophages and manifestations of the functional activity of these cells.

REFERENCES

1. ALLISON, A. C., YOUNG, M. R.: In DINGLE, J. T., FELL, H. B. (eds): *Lysosomes in Biology and Pathology*. North-Holland Publ. Co., Amsterdam 1969. Vol. 2, p. 600.
2. DINGLE, J. T., BARRETT, A. J.: *Biochem. J.* **105**, 19 (1968).
3. ИСИНА, X. M., КОРН, М. Я.: *Бюл. экс. биол. мед.* **25** (9), 83 (1973).
4. WOLF, M. K., ARONSON, S. B.: *J. Histochem. Cytochem.* **9**, 22 (1961).
5. ALLISON, A. C., MAGNUS, I. A., YOUNG, M. R.: *Nature (Lond.)* **209**, 874 (1966).
6. BASTOS, A. L., TERRINHA, M. A., VICÁRIO, J. D., MOURA-NUNES, P. J. L., NUNES-PETISCA, J. L.: *Exp. Cell Res.* **42**, 84 (1966).
7. КОРН, М. Я., ГРИГОРЬЕВА, М. А.: *Proc. 15th Meeting of Epidemiology and Microbiology U. S. S. R. Moscow 1970*. p. 161.
8. БОНДАРЕНКО, В. М., ПЕТРОВСКАЯ, В. Г., КОРН, М. Я., ГАДОШЕВИЧ, В. Н.: *Ж. микробиол.* **11**, 86 (1973).
9. COHN, Z. A., BENSON, B. J.: *J. exp. Med.* **121**, 153 (1965).
10. COHN, Z. A., BENSON, B. J.: *J. exp. Med.* **121**, 835 (1965).
11. COHN, Z. A.: *Properties of Macrophages*. In WILLIAMS, R., FUDENBERG, H. (eds): *Phagocytic Mechanisms in Health and Disease*. Thieme, Stuttgart 1972. p. 39.
12. BARKA, T., ANDERSON, P. J.: *J. Histochem. Cytochem.* **10**, 741 (1962).
13. ФЕДИН, Л. А., БАРСКИЙ, И. Я.: *Микрофотография*. Наука, Ленинград 1971.
14. БАРСКИЙ, И. Я., ХАВКИН, Т. Н.: *ЦИТОЛОГИЯ* **10**, 1501 (1968).
15. ПАПАЯН, Г. В., ИОФФЕ, В. А., ВИНОВАДОВА, Г. Н., БАРСКИЙ, И. Я.: *Цитология* **16**, 395 (1974).
16. ROBBINS, E., MARCUS, P. I.: *J. Cell Biol.* **18**, 273 (1963).
17. ALLISON, A. C., MALUCCI, L.: *J. exp. Med.* **121**, 463 (1965).
18. KOENING, H. J.: *J. Cell Biol.* **19**, 87a (1963).
19. ZELENIN, A. V.: *Nature (Lond.)* **212**, 425 (1966).
20. ROBBINS, E., MARCUS, P. I., GONATAS, N. K.: *J. Cell Biol.* **21**, 49 (1964).
21. NICHOLS, B. A., BAINTON, D. F., FARQUHAR, M. G.: *J. Cell Biol.* **50**, 498 (1971).
22. FISHMAN, M., ADLER, F. L.: In VAN FURTH, R. (ed.): *Mononuclear Phagocytes*. Blackwell, Oxford 1970. p. 581.
23. WALKER, W. S.: *Immunology* **26**, 1025 (1974).

Address of the authors:

T. N. KHAVKIN, I. YA. SAKHAROVA
Institute for Experimental Medicine
Kirov-Prospect 69/71, 197022, Leningrad, U. S. S. R.

I. S. FREIDLIN, V. A. IOFFE
First Medical Institute I. P. Pavlov
Leningrad, U. S. S. R.

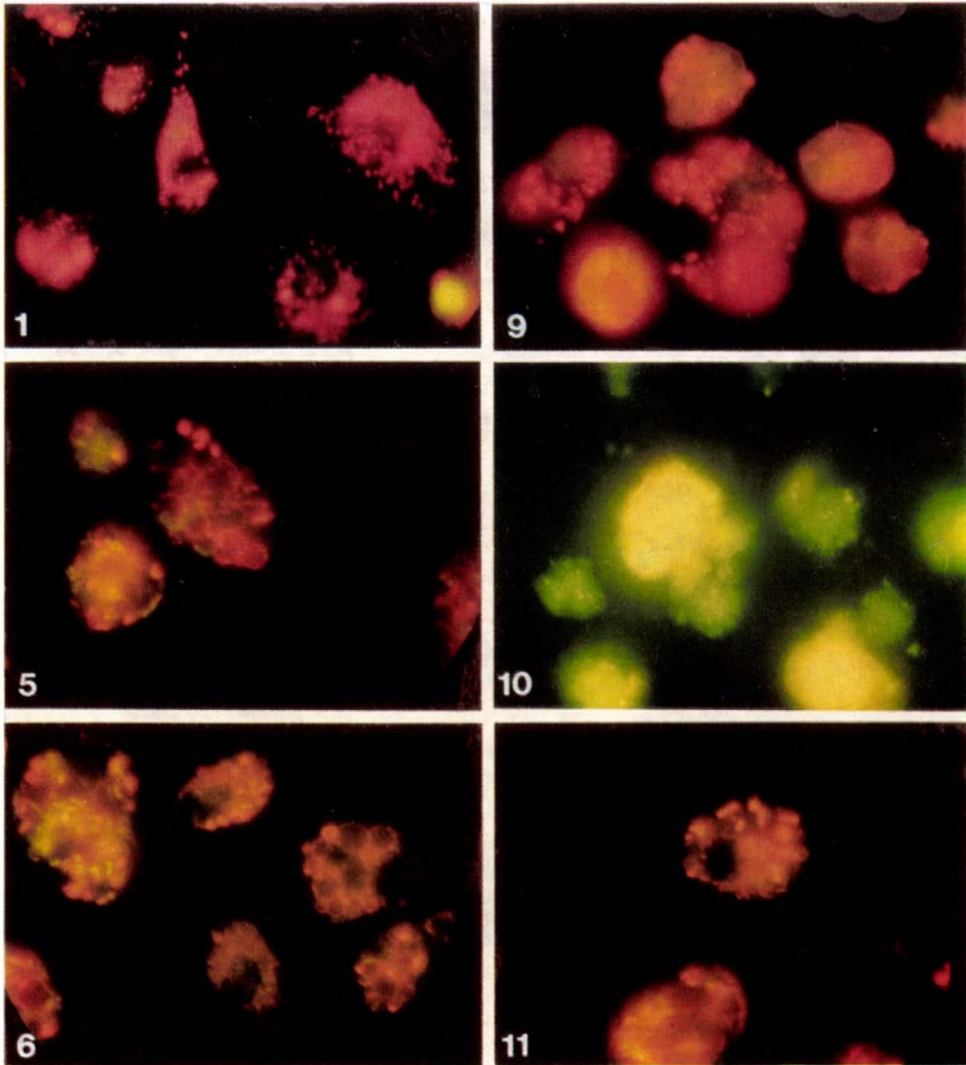


Fig. 1. (KHAVKIN *et al.*). Cultured mouse peritoneal macrophages stained vitally with quinacrine, showing red fluorescence in the lysosomes but not in the nuclei. $\times 120$

Figs. 5. and 6. (FREIDLIN *et al.*). Cultured macrophages 3 hr after uptake of quinacrine and 2 hr after challenge with large dose of *E. coli*. Loss of red granules, red pinocytotic vacuoles. Bacteria show green, orange and red fluorescence

Fig. 9. (FREIDLIN *et al.*). Cultured macrophages 7 hr after uptake of quinacrine, 6 hr after challenge, showing red fluorescence of pinocytotic vacuoles and distorted bacteria

Fig. 10. (FREIDLIN *et al.*). Remnant of cell layer 24 hr after challenge, 1 hr after uptake of quinacrine; loss of red granules, green fluorescence of cells and bacteria

Fig. 11. (FREIDLIN *et al.*). Macrophage 3 hr after challenge with a low dose of *E. coli*; 4 hr after uptake of quinacrine. All ingested bacteria show red fluorescence; red granules are preserved

VITAL FLUORESCENCE MICROSCOPY OF LYSOSOMES IN CULTURED MOUSE PERITONEAL MACROPHAGES DURING THEIR INTERACTIONS WITH MICROORGANISMS AND ACTIVE SUBSTANCES

II. INTERACTIONS OF MACROPHAGES WITH A NON-PATHOGENIC STRAIN OF ESCHERICHIA COLI

I. S. FREIDLIN, T. N. KHAVKIN, N. K. ARTEMENKO and I. YA. SAKHAROVA

*Institute of Experimental Medicine of the U. S. S. R. Academy of Medical Sciences and First
Medical Institute I. P. Pavlov, Leningrad, U. S. S. R.*

(Received December 15, 1976)

Interactions between *Escherichia coli* and cultured macrophages with vitally stained lysosomes have been investigated. The changes in the fluorescence picture of macrophages after ingesting bacteria provide adequate means to establish the fate of quinacrine labelled lysosomes and the responses of the cell. Challenge of macrophage cultures by *E. coli* after preliminary quinacrine staining (2 µg/ml) causes fluorescence of ingested bacteria. Extracellular organisms are not stained. Living or killed bacteria after ingestion show green fluorescence. The reddening of bacteria indicates a contact with labelled lysosome enzyme after lysosome-phagosome fusion. The morphological manifestation of successive stages of bacterial degradation and cell damage in vitally stained macrophage culture is discussed.

Vital staining of macrophage cultures with aminoacridine dyes at low concentration reveals lysosomes which appear as red fluorescent granules. It has been shown [1] that these granules give a positive acid phosphatase reaction and are also revealed by Pappenheim staining. They were shown to vary in size, fluorescence intensity and degree of azurophilia; a particular type of granules is usually predominant in different cells. KORN and GRIGORJEVA [2] and BONDARENKO *et al.* [3] observed that microorganism ingested by acridine orange-stained cells initially showed a green fluorescence which after some time turned into a red one. No explanation of this phenomenon has been offered, nor has the fate of red granules in the cells containing microorganisms been followed. No data are available on long-term studies of interactions between microorganisms and cultured cells stained with aminoacridines. Therefore, it is not clear to what extent macrophage cultures with fluorochrome-treated lysosomes may be employed for vital studies on the dynamics of the parasite-cell interactions. A vital study, a bacteriological study of cells and medium, and microscopy of fixed preparations stained by conventional methods were required to solve these problems. The present investigation is concerned with such an approach: it deals with the interaction of macrophages with non-pathogenic *Escherichia coli* B. which cannot replicate in these cells.

Materials and methods

Bacterial strain. Twenty-four hour old broth cultures of *E. coli* B, either live or killed by formaldehyde vapour, were used in the experiments.

Macrophage culture and procedures. Cultured mouse peritoneal macrophages were used. Staining of their lysosomes with quinacrine, staining procedures, assays for acid phosphatase reaction and fluorescence microscopy of cultures were carried out as described earlier [1]. A 72 hour old macrophage culture maintained on glass coverslips in Leighton tubes was inoculated with 1 ml of suspension of the microorganisms in medium 199, containing 15% calf serum, 100 μ g penicillin and 10 μ g streptomycin. The organism concentration was raised to 100 or 10 bacterial cells per macrophage (10^8 or 10^7 bacteria per ml).

Bacteriological study and microscopy. The culture medium, the homogenates of cells washed in 10 portions of the medium as well as the 10th portion of the washing fluid were examined 5, 15, 30 min, 1, 1.5, 2, 4, 6, 24 and 48 hr after infection. A vital study of macrophages and examination of fixed stained preparations were carried out at the same intervals.

Fluorochrome treatment. Macrophage cultures intended for a vital study at intervals of 15 min to 6 hr were stained with quinacrine 1 hr prior to infection. Macrophage cultures to be examined 24 and 48 hr after inoculation were treated with fluorochromes 1 hr before the commencement of the study.

A study of the interaction of cultured macrophages with 1.4 μ m latex spheres was conducted as control. Examination was carried out 2, 6 and 24 hr after the introduction of latex into the culture medium.

Results

Experiments with live *E. coli* B. Bacteriological study. The study of macrophage homogenates showed that 10^6 – 10^5 bacteria become somehow connected with the cells after 30 min of incubation, the initial concentration being 10^8 – 10^7 bacteria in 1 ml per 10^6 macrophages. It was impossible to establish what fraction of these bacteria was ingested and what fraction was adsorbed, since all non-ingested microorganisms were not removed even after the cell layer had been washed ten times. The organism concentration in the 10th portion of the washing fluid ranged from 10^5 to 10^3 bacteria/ml, depending on the original concentration (10^8 – 10^7).

The bacterial counts in the culture medium containing the above antibiotics, showed a gradual decrease. From the initial level of 10^7 – 10^8 per ml, it fell to 10^3 – 10^4 after two hours of incubation and was 10^2 and 10^4 after 24 hr.

Microscopy of fixed preparations. When a culture was inoculated with a sufficiently large dose (100 bacteria per 1 macrophage), 100% of these cells took up microorganisms. The level of saturation, which averaged $2.3 \pm 0.8 \times 10^7$ per 10^6 cells, was reached within the first two hours. Azurophilic grains, revealed in macrophages by Pappenheim staining disappeared quickly; cells with predominant "large" grains took up microorganisms at a slower rate than the other cells (Fig. 1a, b).

On saturation with bacteria, macrophages gradually became rounded and were rich in vacuoles (Fig. 1c). The ingested bacteria were deformed. After 24 hr the cell layer was destroyed and most of macrophages were necrotized (Fig. 1d). Viable macrophages contained about 7 bacteria per cell which may be accounted for by an additional ingestion of organisms from the nutrient medium.

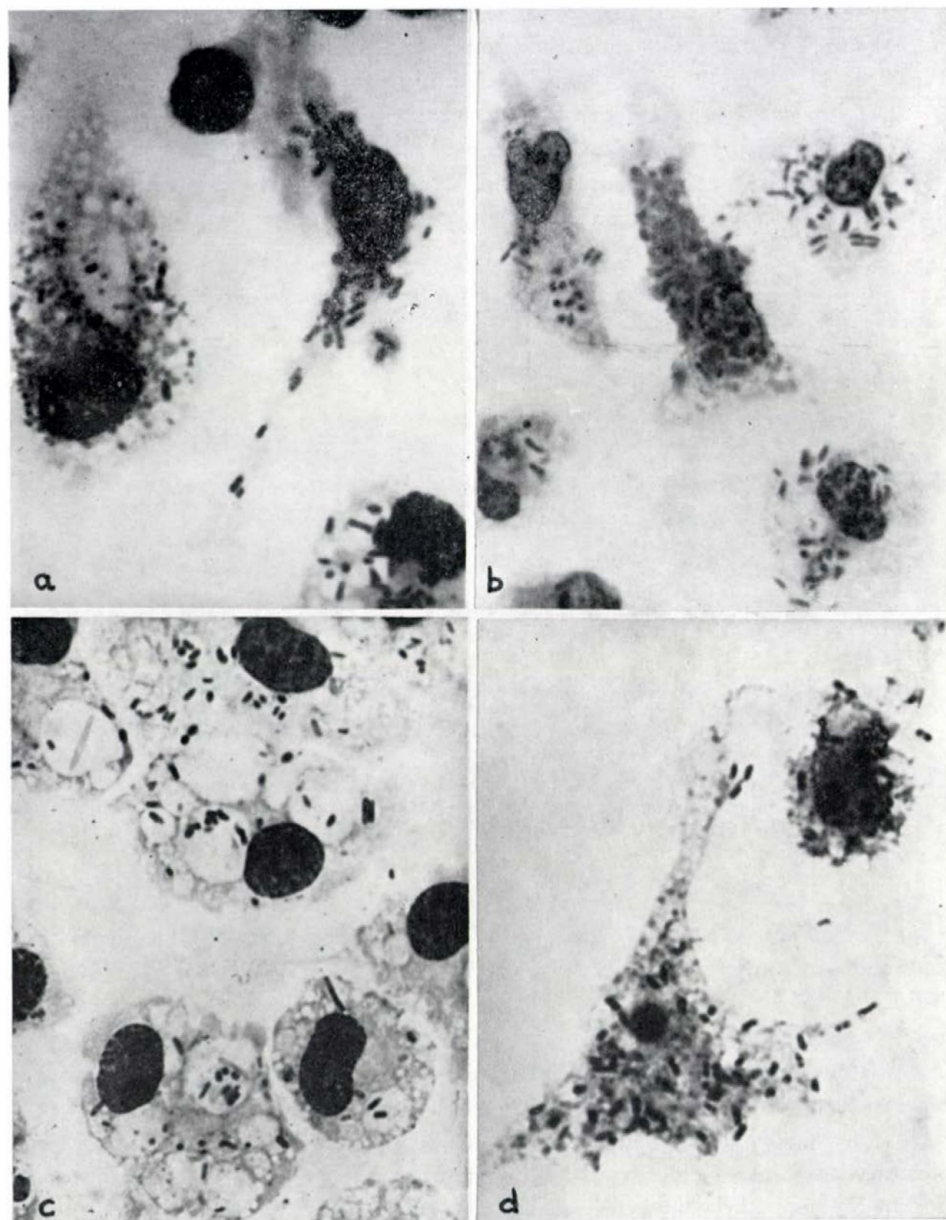


Fig. 1. Stages of bacteria-macrophage interaction after challenge with *E. coli* B: (a) 1 hr after challenge, most macrophages with ingested organisms are deprived of azurophilic granules, one cell preserves the medium-size azurophilic granules; (b) 2 hr, identical picture, showing a cell with well-preserved large granules; (c) 6 hr; macrophage vacuolation; deformation and poor staining of some ingested bacteria; (d) 24 hr, dead cell is infiltrated with bacteria. Pappenheim stain, $\times 1200$

Changes in the acid phosphatase reaction indicated a pronounced loss of the enzyme. Phosphatase accumulated around the ingested microorganisms (Fig. 2a). At 24 hr, its traces could be found in few cells only.

At lower doses of infection (10 bacteria per macrophage), only 50–80% of the cells ingested bacteria. The concentration was $6.4 \pm 3.0 \times 10^6$ bacteria per 10^6 macrophages two hours after infection. Fewer azurophilic grains disappeared in the macrophages. The decline in acid phosphatase activity was

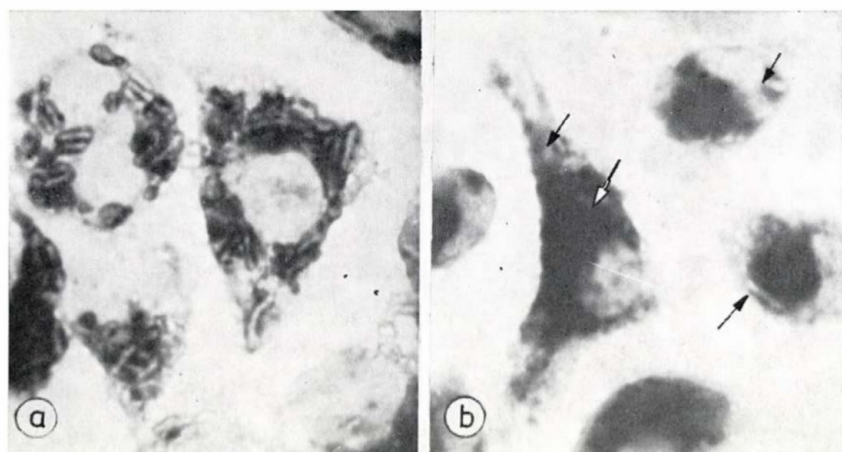


Fig. 2. Acid phosphatase reaction 2 hr after challenge: (a) the cells have taken up many bacteria and show diminished enzyme activity, phosphatase accumulates around the ingested organisms; (b) cells with single bacteria (arrows) and showing usual enzyme content. $\times 1178$

insignificant (Fig. 2b). At 24 hr, the cell layer was preserved. Organisms could be detected in not more than 20% of the cells. Their count per cell decreased 13-fold. At 48 hr, the culture was virtually free from microorganisms and normal in appearance.

Vital fluorescence microscopy. Ingested and non-ingested microorganisms were well distinguishable, since the latter showed no fluorescence (Fig. 3), while ingested organisms showed a green fluorescence. The interactions between microorganisms and cells were best visualized by a comparison of the results of fluorescence and Pappenheim staining (Fig. 4).

At hours 2–4, macrophages were saturated with green, orange and red fluorescent organisms (colour plate, Fig. 5). Bacteria fluorescing in red were more distinguishable than green ones under the phase-contrast microscope.

Fluorescent organisms in the cells distort the assessment of lysosome content on the basis of the total fluorescence of macrophages. Visual observation showed that the population content of cells with a great number of red granules

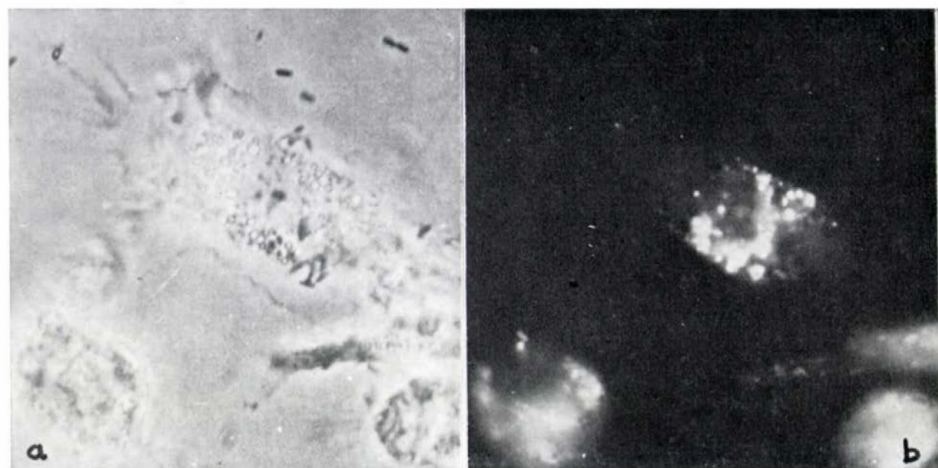


Fig. 3. Phase-contrast (a) and fluorescent (b) images of the same cells, 2 hr after uptake of quinacrine, 1 hr after challenge; extracellular bacteria are distinguished by phase contrast only; ingested bacteria show fluorescence but are hardly distinguished by phase contrast. $\times 600$

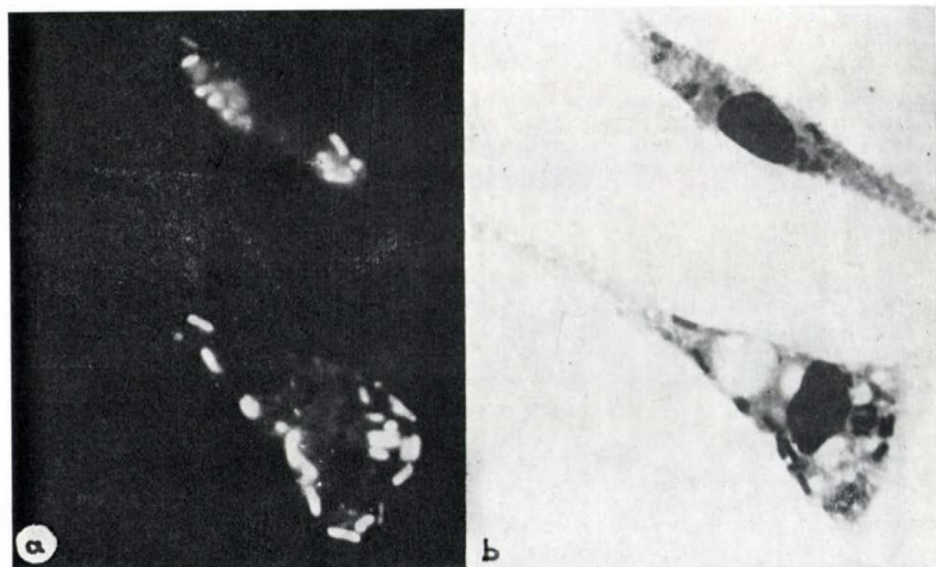


Fig. 4. Two pictures of the same infected cells. (a) Fluorescent image after uptake of quinacrine, showing bacteria and some red granules; (b) light photomicrograph after Pappenheim stain, showing cell structures and bacteria. In both pictures all ingested bacteria are well distinguished. $\times 1200$

decreased from 45–50 to 5–10% by hour 2. There were few macrophages containing considerable numbers of large red grains, alongside with cells in which almost no red granules could be seen (colour plate, Figs 5 and 6). The former ingested organisms at a lower rate than the latter, and some ingested organisms still

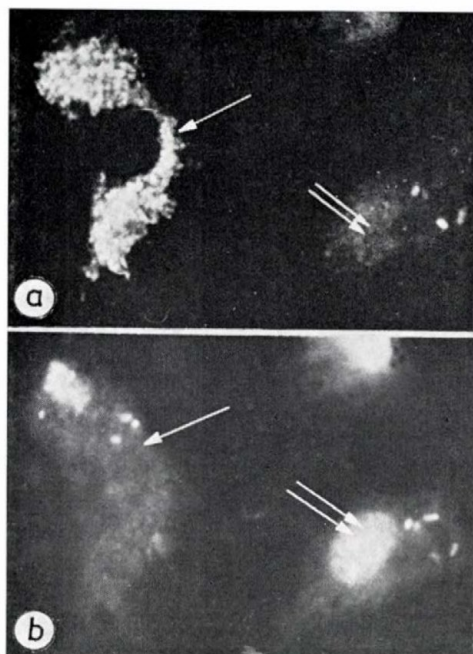


Fig. 7. Photomicrographs of selective red (a) and green (b) fluorescence in the same infected cells: one cell (arrow) contains many large red granules and some bacteria with green fluorescence; another cell (double arrow) losing red granules, shows green fluorescence of its nucleus, and green and red fluorescence of ingested bacteria. $\times 600$

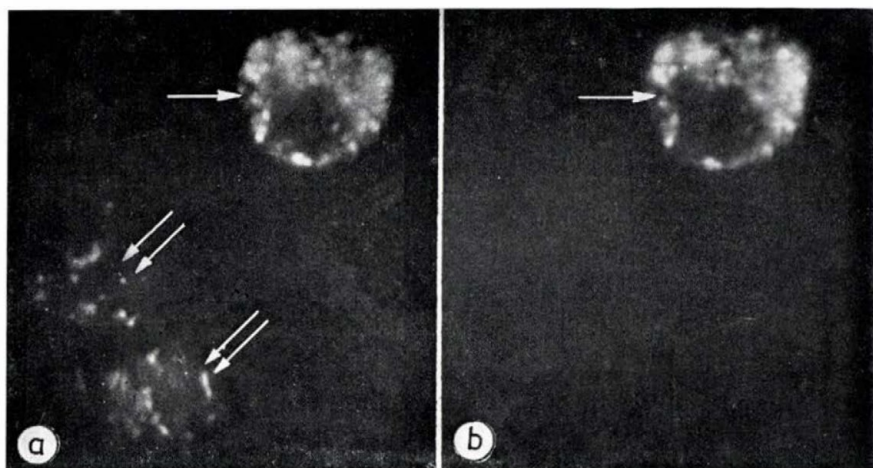


Fig. 8. Fluorescent photomicrographs of the same cells with colour selection. (a) Picture made with standard barrier filter, showing a cell with many red granules (arrow) and two cells (double arrows) deprived of red granules and containing green fluorescent bacteria; (b) selective red fluorescence. $\times 600$

showed green fluorescence (Fig. 7). The intensity of green fluorescence of nuclei and cytoplasm was particularly high in those cells where all red granules disappeared at a fast rate. Microorganisms inside such cells continued to show a green fluorescence.

While vacuoles with red fluorescent contents were infrequent in normal cell cultures [1], such vacuoles showed a sharp increase in number after the onset of ingestion. Microorganisms in these vacuoles appeared as shapeless red clusters (Colour plate, Fig. 9). Moreover, unaltered red and green fluorescent bacteria occurred in macrophages, which seemed to be a manifestation of the ongoing process of ingestion. By hour 24, cells without red granules and with nuclei and cytoplasm fluorescing green predominated among the remaining macrophages (Fig. 10). The red granule content of such cells was half that of the control.

When the macrophage culture was infected with a low dose (10 microorganisms per one macrophage), the changes in the cells containing microorganisms were much the same, only less pronounced. By hour 2, the red granule content in macrophages decreased 1.2-fold, the cells contained red and green bacteria at that stage. The proportion of red bacteria began to increase after 4–6 hr. Many macrophages contained only bacteria fluorescing red during this period (colour plate, Fig. 11).

There were no microorganisms in most macrophages at hour 24. The number of red granules was close to the original level. There were, however, cells without granules with nuclei and cytoplasm fluorescing green (Fig. 12). After 48 hr, no microorganisms were seen in the macrophages.

Experiments with killed E. coli B. Prior to the study of interactions of microorganisms with macrophages, an experimental treatment of a suspension of killed *E. coli* B with quinacrine at concentrations up to 2 µg/ml was carried out. Green fluorescence of microorganisms was recorded after one hour incubation, red fluorescence did not occur.

Large doses (100 microorganisms per one macrophage) were placed in test tubes containing macrophages. Killed bacteria were taken up at a slower rate than viable ones. The maximum quantity of ingested bacteria amounted to $7.0 \times 10^6/10^6$ macrophages after 2 hr. This was followed by a gradual decrease in the cell content of bacteria. No bacteria were observed in macrophages after 24 and 48 hr. No damage to the cells was recorded.

Vital fluorescence microscopy revealed changes similar to those involved in macrophage infection with live *E. coli* B, although they were less pronounced. The proportion of cells with numerous red granules decreased 1.5 times after 2 hr. The gradual changes in fluorescence from green to red were more apparent with killed than live bacteria. The red granule content was restored after 24–48 hr and no microorganisms occurred in the cells while unlike in control cultures, the nuclei of many macrophages showed a green fluorescence.

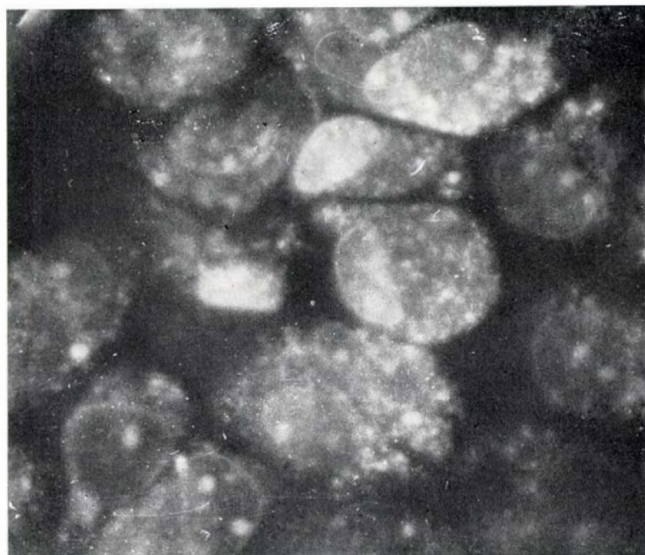


Fig. 12. Cultured macrophages 24 hr after challenge with a low dose of bacteria, 1 hr after uptake of quinacrine, showing red granules and green fluorescence of nuclei of varying intensity

Control. A suspension of latex particles (100 spheres per macrophage) was placed into the culture medium, following fluorochrome treatment of macrophages. Latex particles were taken up by macrophages during the first hour. No loss of acid phosphatase, even from the cells containing numerous spheres, was observed. Vital microscopy did not reveal any noticeable loss of red granules or fading of the green fluorescence of macrophage nuclei. Some latex spheres had a rim of red fluorescence.

Discussion

A study employing bacterial inoculation and staining techniques showed that the interactions between non-pathogenic *E. coli* B and cultured mouse peritoneal macrophages involve a rapid ingestion of microorganisms followed by their digestion. Ingestion of large quantities results in damage and, eventually, death of macrophages, which seems to be due to the action of endotoxins released from microorganisms. When a small proportion of live or dead *E. coli* are ingested, macrophages appear to be able to stand the damage. Non-toxic latex does not affect the macrophages, even when large numbers of latex spheres are ingested.

Vital fluorescence microscopy supplements the results of studies involving the use of bacterial inoculation and staining techniques. It supplies a

means for identifying intra- and extracellular microorganisms, for following the fate of ingested bacteria, their interactions with lysosomes in living cells and for detecting the earliest manifestations of cell damage.

Unlike intact microorganisms, ingested bacteria show fluorescence. Their initial green fluorescence gradually turns into a red one. Our results suggest that the reddening of organisms may be regarded as a morphological manifestation of their interaction with the lysosomal contents labelled with quinacrine. There is another approach which seems to be less likely: microorganisms become red as a result of a re-distribution of quinacrine which leaves the lysosomes and stains the dying bacteria (STRUGGER's effect [4]). This is at variance with our observation that a concentration of 2 μg quinacrine per 1 ml of medium was sufficient to produce a red fluorescence of lysosomes in living cells but it was not enough to induce a red fluorescence of killed bacteria.

Our results suggest the following possible interpretation of fluorescence patterns. On delivery of stained enzymes to phagosomes, a rim of red fluorescence appears around the edges of ingested microorganisms. Subsequently, the whole bacterial cell becomes red. The red fluorescent rim corresponds to that produced by neutral red in the experiments of COHN and BENSON [5]. This represents a gradual concentration of hydrolases around the microorganism. A gradual conversion of the green fluorescence of microorganisms to a red one is particularly vivid in experiments with killed *E. coli*.

As the microorganisms become red, all or some red granules (depending on the number of ingested bacteria) disappear from the macrophages. When the saturation of cells with microorganisms is sufficiently high no lysosomes are left in the cells and, therefore, some bacteria remain green. NORTH and MACKANESS [6] reported the exhaustion of cell lysosomal material on the basis of an electron microscopy study.

The appearance of numerous vacuoles showing red fluorescence in macrophages containing *E. coli* may further be accounted for by a delivery of lysosomal contents to pinocytotic vacuoles. This is supported by a much greater incidence of such vacuoles during microorganism ingestion than in "normal" macrophage cultures [1].

In addition to the delivery to phagosomes, the contents of the lysosomes of macrophages which had ingested microorganisms may leave the cell and enter the medium. This would provide another explanation of the disappearance of red granules. This is indirectly supported by the results of TAVERNE *et al.* [7]. According to their data, when a small number of *E. coli* are ingested, lysosomal enzymes do not enter the medium, because the cells can counteract the effect of a small quantity of toxic substances. When a great many microorganisms are ingested, lysosomal enzymes leave into the medium, thus indicating cell damage. Our morphological data are consistent with these biochemical results.

The rate of degranulation as well the reddening of ingested bacteria in the cells containing numerous large granules showing bright red fluorescence is lower than in the other macrophages. This suggests a lower lability of lysosomes in such macrophages and is another indication of the heterogeneity of cultured peritoneal macrophages described by us earlier [1].

The green fluorescence of the nuclei and, subsequently, of the cytoplasm of many macrophages should be interpreted as a morphological manifestation of cell damage caused by the *E. coli*. The phenomenon occurs in cells containing *E. coli* only; it becomes more apparent as bacteria are digested and does not take place when latex spheres are ingested. The effect is similar to that observed after prolonged exposure of fluorochrome-treated cells to exciting light [1]. It allows an assessment of cell damage to be made before it can be detected by other means.

Hence, studies of the fluorescence of cultured macrophages after they have taken up *E. coli* provide an adequate means of establishing the fate of lysosomes and the responses of the cell during its interactions with microorganisms. Application of vital fluorescence microscopy is a substantial contribution to the methods of study of the parasite-cell system *in vitro* and it may effectively be used in conjunction with other procedures.

Addendum. After preparing and submitting this manuscript we received a paper by GOREN *et al.* [8] who observed a progressive coloration of ingested yeast cells with acridine orange (at first green and later red) in the macrophages with prelabelled lysosomes. They explain the colour change by an accumulation of pin-pointed lysosomal content in the phagolysosomes, in good agreement with our data.

REFERENCES

1. KHAVKIN, T. N., FREIDLIN, I. S., SAKHAROVA, I. YA., IOFFE, V. A.: *Acta microbiol. Acad. Sci. hung.* **24**, 111 (1977).
2. KORN, M. YA., GRIGORIEVA, M. A.: *Proc. 15th Meeting of Epidemiology and Microbiology U. S. S. R. Moscow 1970*, p. 161.
3. БОНДАРЕНКО, В. М., ПЕТРОВСКАЯ, В. Г., КОРН, М. Я., ГАДОШЕВИЧ, В. А.: *Ж. микробиол.* **11**, 86 (1973).
4. STRUGGER, S.: *Naturwissenschaften* **34**, 267 (1947).
5. COHN, Z. A., BENSON, B. J.: *J. exp. Med.* **121**, 835 (1965).
6. NORTH, R. J., MACKANESS, A.: *J. Ultrastruct. Res.* **16**, 96 (1966).
7. TAVERNE, J., BLYTH, W. A., BALLARD, R. D.: *J. Hyg. (Lond.)* **72**, 297 (1974).
8. GOREN, M. B., D'ARCY HART, P., YOUNG, M. R., ARMSTRONG, J. A.: *Proc. nat. Acad. Sci. (Wash.)* **73**, 2510 (1976).

Address of the authors:

T. N. KHAVKIN, I. YA. SAKHAROVA
Institute for Experimental Medicine
Kirov-Prospekt 69/71, 197022, Leningrad, U. S. S. R.

I. S. FREIDLIN, N. K. ARTEMENKO
First Medical Institute I. P. Pavlov
Leningrad, U. S. S. R.

EFFECT ON PLANTS OF SERA FROM PATIENTS WITH HEPATITIS

J. BUDAI, BEATRIX GYÖRGY, GY. SZÉCSEY and KATALIN BERTA

“László” Central Hospital for Infectious Diseases, Budapest and Horticultural Research Institute,
Budapest

(Received December 20, 1976)

Serum samples from 42 patients with acute viral hepatitis (23 HB_sAg-positive and 19 HB_sAg-negative) and from seven healthy subjects were inoculated into leaves of various plants. Lesions developed in three-quarters of *Nicotiana sylvestris* and *Nicotiana langsdorffii* leaves inoculated with HB_sAg-positive sera and in a quarter of those inoculated with HB_sAg-negative sera. The changes manifested themselves with yellowing and curliness. Necrosis did not occur. A safe and simple injection technique has been developed for study in plants of human viruses.

Little is known of the interactions between animal viruses and plant hosts. VENDÉC [1] and ÁBRAHÁM [2] were the first to report on human pathogenic viruses causing in plants lesions resembling those caused by plant viruses. The subsequent reports have been contradictory. DASHKEEVA *et al.* [3, 4] induced mosaic-like changes and necroses in petunia and tobacco plants with sera from patients with hepatitis. These authors supposed that the cucumber mosaic virus might have adapted to man and developed into hepatitis virus. Subsequently, COSSART and FIELD [5] and JENSON *et al.* [6] assumed a possible relationship between the cowpea chlorotic mottle virus and the human hepatitis virus. ZUCKERMAN [7] called attention to morphological similarities between the different structural units of the HB_sAg and proteins isolated from isometric plant viruses.

The greatest interest has been evoked by the studies of BANATVALA and PAYNE [8, 9]; these authors succeeded in inducing characteristic lesions, *viz.*, yellowing and necrosis in tobacco leaves (*Nicotiana sylvestris*) with serum samples from patients suffering from HB_sAg-positive hepatitis. The pathological process could be prevented by pretreatment of the samples with heat or hepatitis convalescent sera. Similar experiments with serum samples from patients with HB_sAg-negative acute hepatitis or jaundice of non-hepatic origin led to negative results.

These surprising results prompted several research groups to initiate similar experiments. BEASLEY and SU [10] as well as HOWARD and MCCARTHY [11] failed to reproduce the results, but PAYNE and BANATVALA [12] questioned the reliability of their experimental technique. SÄNGER [13] and ESANU and BABES

[14] succeeded in reproducing the results in 20–60% of the cases, while DHALI-WAL *et al.* [15] published unsuccessful experiments.

We have made efforts to reproduce the experiments of BANATVALA and PAYNE [8, 9], also on plants other than *N. sylvestris*. In addition, we have developed a new technique for inoculating plants with viruses.

Materials and methods

Serum samples from 42 patients with acute hepatitis were collected. HB_sAg was assayed by the microcomplement-fixation test in the National Institute of Hygiene, Budapest. The results of these tests were still unknown when plants were inoculated with the sera. Of the acute hepatitis cases 23 were positive for HB_sAg; in 16 cases the antigen titre was higher than 1 : 20. Four serum samples were also anticomplementary, suggesting the presence of antigen-antibody complex.

Serum samples from 7 HB_sAg-negative healthy subjects served as control.

Plants. Besides *N. sylvestris*, the following plants were inoculated: *N. langsdorffii*, *N. clevelandii*, *N. rustica*, *Chenopodium quinoa*, *Ch. foetidum*, *Zinnia elegans*, lettuce and cucumber. Several plants were inoculated with each serum and the inoculations were repeated four times. The tobacco plants were 6 weeks old when inoculated. After being inoculated the plants were kept in the Hospital's greenhouse during a 40 days observation period.

Initially, the inoculations were performed as usual in plant virology, i. e., serum samples pretreated with carborundum were rubbed in with fingers protected by rubber gloves. Later, we developed a method which has proved more exact. We injected 0.1–0.3 ml serum into the veins of leaves, using a tuberculin syringe.

The reaction was considered positive when extensive colour changes, yellowing and curling, occasionally chlorotic spots and/or mosaic-like lesions were observed in the inoculated leaves.

Results

HB_sAg-positive sera. The rate of positive leaves was the highest when HB_sAg-positive sera were tested in *N. sylvestris* or *N. langsdorffii*, viz., three-quarters of the leaves developed lesions. As to the other plants, more than 10 sera were inoculated into each of *Ch. quinota* and *Z. elegans* plants. For these plants, the rates of positive reactions were much lower (Table I).

HB_sAg-negative sera. *N. sylvestris* and *N. langsdorffii* leaves were inoculated with 15 sera. Lesions were induced by 6 and 2 samples, respectively.

The lesions appeared as yellowing and curling of the leaves 2–5 days after inoculation. Necrosis was never observed. Occasionally, chlorotic spots were seen in *N. rustica* leaves. Several *Nicotiana* leaves became yellow *in toto* and stunted as compared with the controls.

There was no appreciable difference between the positive reaction of infected leaves and those inoculated by rubbing in of the serum. However, the injection technique is simpler and the risk of laboratory infection is lower.

Sera from healthy subjects caused no lesions on any of the inoculated leaves.

Table I

Response of plants to inoculation with serum samples from patients with acute hepatitis and those from healthy subjects

Plant species	Sera from hepatitis		Sera from healthy subjects
	HB _s Ag positive	HB _s Ag negative	
<i>Nicotiana sylvestris</i>	17/19*	6/15	0/6
<i>N. langsdorffii</i>	12/20	2/15	0/4
<i>N. rustica</i>	1/2	1/2	0/2
<i>N. clevelandii</i>	0/3	0/1	—
<i>Chenopodium foetidum</i>	2/4	0/4	0/2
<i>Ch. quinoa</i>	4/13	2/8	0/2
<i>Zinnia elegans</i>	4/12	1/7	0/2
<i>Lactuca sativa</i> (lettuce)	2/3	0/1	—
<i>Cucumis sativus</i> (cucumber)	2/3	0/1	—

*Positive/inoculated

Discussion

Our observation that lesions developed in about 75% when *N. sylvestris* or *N. langsdorffii* leaves were inoculated with HB_sAg-positive sera seems to agree with the observations of BANATVALA and PAYNE [8, 9]. However, necrosis never developed in the leaves inoculated by us. The lack of necrosis might be attributed to the fact that we could not secure all the environmental factors prescribed by the mentioned authors. Another inconsistency is that lesions were induced in tobacco leaves by a quarter of our HB_sAg-negative hepatitis sera.

It cannot be excluded that the lesions observed by us were induced not by the hepatitis B virus itself, but by toxic substances present in HB_sAg-positive sera frequently or at high concentration.

Further investigations are necessary to elucidate the causes of the contradictions.

REFERENCES

1. VENDÉG, V.: Rev. med. (Trîgu-Mureş) **3**, 32 (1948).
2. ÁBRAHÁM, A. S.: Z. ges. inn. Med. **18**, 720 (1958).
3. ДАШКЕЕВА, К. Н., СПАТАРЕНКО, С. С., ВЕТРОВА, Ф. М.: Вирусологические исследования на Дальнем Востоке, Владивосток, 21, 1969.
4. СПАТАРЕНКО, С. С., ДДШКЕСВА, К. Н.: Изв. Ан. Молд. ССР Кишинев **9**, 56 (1967).
5. COSSART, J. E., FIELD, A. M.: Lancet **2**, 848 (1970).
6. JENSON, A. B., MCCOMBS, R. M., SAKURADA, N., MELNICK, J. L.: Exp. molec. Path. **13**, 217 (1970).

7. ZUCKERMAN, A. J.: *Lancet* **1**, 1486 (1973).
8. BANATVALA, J. E., PAYNE, CH.: 14th European Symposium on Poliomyelitis and Other Virus Diseases, Ankara 1973.
9. BANATVALA, J. E., PAYNE, CH.: *Lancet* **1**, 1359 (1973).
10. BEASLEY, P. R., SU, H. J.: *Lancet* **2**, 1203 (1973).
11. HOWARD, C. R., MCCARTHY, D. A.: *Lancet* **2**, 219 (1974).
12. PAYNE, CH., BANATVALA, J. E.: *Lancet* **2**, 1327 (1973).
13. SÄNGER, H.: cit. PAYNE, CH., BANATVALA, J. E.: *Lancet* **2**, 1327 (1973).
14. ESANU, V., BABES, V. T.: *Rev. roum. Virol.* **25**, 134 (1974).
15. DHALIWAL, A. S., RUBINSTEIN, H. M., DIETZ, A. A.: *Lancet* **1**, 1248 (1975).

Address of the authors:

JÓZSEF BUDAI, GYÖRGY SZÉCSÉY, KATALIN BERTA
"László" Central Hospital for Infectious Diseases
Gyáli út 5-7, H-1097 Budapest, Hungary

BEATRIX GYÖRGY
Horticultural Research Institute
Nagytétényi út 198, H-1223 Budapest, Hungary

EXCLUSION OF FALSE POSITIVE REACTIONS DUE TO IgG-BINDING Fc-RECEPTOR(S) IN HERPESVIRUS SEROLOGY

M. SIMON

Hungarian Army Medical Corps, Budapest

(Received January 7, 1977)

The Fc/IgG receptor(s) demonstrated earlier on living cells infected by herpes simplex virus (HSV) continue to function after fixation of the cells in acetone. The receptor(s) reacting with non-virus-specific IgG molecules disturb the immunofluorescence (IF) tests for HSV antibodies of the IgG class. The discrepancy can be eliminated by blocking the Fc receptor sites by pretreating the cell preparations with normal rabbit globulin. The affinity of mammalian IgGs for the Fc receptor varies with the species. An alternative way for the elimination of the discrepancies is the use of *Staphylococcus aureus* protein "A"-FITC conjugate instead of anti-human-IgG FITC. The former as an Fc reagent selectively detects the free Fc end of virus-specific IgG molecules fixed on the cell surface by their Fab end. A staphylococcus rosette test has been evolved for measuring herpesvirus antibodies. The principle of the test is the same as of the IF test with the protein "A" FITC conjugate. This test is simple, sensitive, and suitable for type-specific differentiation of HSV antibodies, especially for the exclusion of non-type specific reactions with HSV-2 in human sera.

Sheep erythrocytes sensitized with homologous rabbit antiserum are adsorbed by cells infected by herpes simplex virus (HSV) [1, 2]. The receptors responsible for the adsorption are in some respects resembling to cytophilic antibody receptors of macrophages [3]. A similar phenomenon has been observed with cells infected by cytomegalovirus (CMV) [4]. The receptors appearing on the infected cells react with the Fc region of IgG molecules [5–7].

We have observed disturbances due to Fc receptors in the course of determining IgG-type HSV antibodies by indirect immunofluorescence. A study was therefore made to detect the IgG receptors responsible for the false reactions and to eliminate the disturbing effect, and thus to increase the specificity of the antibody assay.

Materials and methods

Viruses. Strains Hil and LF of HSV type 1, strains Lov and Bry of HSV type 2 were used.

Cell cultures. The HSV strains were propagated in the cell lines ERK and Vero and in the rhesus monkey cell line III/1 [8]. As maintenance fluid, Parker's 199 enriched with 5% calf serum was used.

Immunofluorescence (IF) antibody assay. Cells of virus-infected cell cultures showing initial cytopathic changes were scraped off from the bottle wall and suspended and washed once in PBS. Eight small drops of the suspension were placed on each slide, allowed to dry at room temperature and fixed in acetone for 10 min. The drops were separated by nail polish.

For titrating virus-specific antibodies in human sera serial serum dilutions were prepared on TAKÁTSY's Microtiter trays [9]. One drop from each dilution was added to a spot of

acetone-fixed cells. The starting serum dilution was 1 : 10 when IgG antibodies and 1 : 5 when IgA or IgM antibodies were determined. The slides were incubated in a wet chamber at room temperature for 30 min when IgG or IgA antibodies, and at 37°C for 3 hr when IgM antibodies, were to be determined. Subsequently, the preparations were washed three times for 5 min in PBS, allowed to dry, and one drop of the working dilution of anti-human-IgG, IgA or IgM FITC conjugate (all prepared from goat serum, Hyland) was added to each. After an incubation at room temperature for 30 min, irrespective of the immunoglobulin class, the preparations were washed three times for 5 min, counter-stained with 1 : 25,000-diluted Evans blue for 2-3 min and examined under the fluorescence microscope.

Sensitized sheep erythrocytes for the detection of Fc receptors. Washed sheep erythrocytes were sensitized with the 1 : 100 dilution of an anti-sheep-erythrocyte rabbit serum (haemolysin) as usual for the complement-fixation test, washed again and diluted to 1% in PBS. The haemolysin preparation used by us agglutinated sheep erythrocytes up to 1 : 400. A 1% suspension of sensitized sheep erythrocytes was used for the detection of protein "A". This protein of about 40 000 dalton molecular weight is an Fc receptor present on the bacterial surface of some *Staphylococcus aureus* strains. Our protein "A" preparation agglutinated the sensitized sheep erythrocytes and the agglutination could be inhibited by means of mammalian IgG preparations.

Detection of Fc (IgG) receptors on HSV-infected cells by S. aureus indicator cells. Protein "A" selectively reacts with the Fc region of the IgGs but not of IgAs and IgMs of mammalian sera [10, 11]. The Fab region of the IgG molecules is accessible on the surface of protein "A"—IgG complexes. We used the Cowan I *S. aureus* strain, which is rich in protein "A". The bacterial suspensions were prepared as described by KRONWALL [12]. In brief, 24 hr cultures were kept in 0.5% formaldehyde solution for 3 hr, then washed three times with, and diluted in 10 volumes of, saline. The 10% suspension was inactivated in a water bath of 80°C for 4-5 min. The suspension thus obtained, with sodium azide added, could be stored at 4°C for several months.

Isolation, and conjugation with FITC, of protein "A". In spite of the formalin and heat treatment, protein "A" tends to detach from the bacterial surface and appears in the medium. The speed of the process positively correlates with the temperature. Solubilized protein "A" was isolated by adsorption to glutaraldehyde-insolubilized human IgG [13] and elution into 0.1 M glycine-HCl buffer of pH 2.8. The protein "A" content of the eluate was measured by agglutination of sensitized sheep erythrocytes. The eluate that contained 0.5 mg/ml protein and agglutinated the erythrocytes up to 1 : 8000, was conjugated with FITC as usual. The free dye was removed by gel filtration on Sephadex G-25 columns. Satisfactory labelling required repeated conjugation. The undiluted filtrate was used in the test.

Preparation of staphylococcus indicator cells. Formalin-treated and heat-fixed staphylococcus suspension was conjugated with FITC. The surface-fixed protein "A" is capable of reacting with IgG molecules. HSV infected cell suspensions were incubated with anti-HSV serum, washed, and mixed with the staphylococcus suspension labelled with FITC (St-FITC). If the corresponding viral antigen was present on the cell surface, the bacterial cells attached to the free Fc region of the IgG molecules specifically bound on the cell surface. The cell surface became covered by fluorescent staphylococci, which formed a characteristic rosette. The contrast to the negative cells was enhanced by counter-staining with Evans blue. Similarly to the membrane IF technique, the antiviral IgG in sera can be titrated by this method with high sensitivity.

Results

Indirect demonstration of Fc (IgG) receptors on HSV-infected cells. We have often observed false positive IgG reactions when human sera negative for HSV antibodies (as tested by neutralization, complement fixation and passive haemagglutination methods) were examined. Similar discrepancies did not occur when sera were tested for IF antibodies of the IgA or IgM type. The false positive reaction was independent of the origin of the cells (Vero, HeLa, ERK, etc.).

We assumed that the anomalies in the IF test were due to the appearance on the surface of HSV-infected cells of sites (receptors) capable of adsorbing

non-virus-specific IgG molecules. To eliminate the anomalies, we attempted to block the sites responsible for the non-specific adsorption of globulins by pretreating the cells with allogeneic normal globulins.

For this purpose, the globulin fraction was prepared by precipitation with ammonium sulphate, from normal calf and rabbit sera. Acetone-fixed HSV-infected cell preparations were incubated for 30 min at room temperature with a solution containing 10 mg of the globulin fraction in 1 ml. The unbound globulin was removed and HSV-positive and negative human sera were tested for IF IgG antibodies.

Table I shows that the negative human sera elicited no IF when tested with HSV cell preparations pretreated with normal rabbit globulin. The same sera gave positive IF reaction with the preparation without pretreatment and with that pretreated with calf globulin. The specific titres of the anti-HSV-positive sera were lower with the preparation pretreated with rabbit globulin than with the untreated or calf globulin-pretreated ones, but the specific reaction never disappeared. It seems therefore reasonable to suppose that, like the living HSV-infected cells, those fixed with acetone react, besides virus-specific antibodies, with other globulins, supposedly of IgG nature. The fixation of the non-specific globulin seemed to be of higher degree on cells infected by HSV type 1 than on those infected by HSV type 2.

Table I

Non-specific adsorption of IgGs of human sera to acetone-fixed preparations of cells infected by HSV-1 or HSV-2 and inhibition of the adsorption by pretreatment of the cells with heterologous serum globulins

Human sera	IgG IF antibody titres determined					
	without pretreatment		after pretreatment with			
			calf globulin		rabbit globulin	
	in cell preparations infected by					
	HSV-1	HSV-2	HSV-1	HSV-2	HSV-1	HSV-2
HSV-antibody negative sera*	80	10	80	10	<5	<5
	160	20	160	20	<5	<5
	80	10	80	10	<5	<5
HSV-antibody** positive sera	>640	160	>640	160	160	80
	640	80	640	80	320	80
	640	160	640	160	160	80

* Sera negative with complement fixation, passive haemagglutination and neutralization tests

** Sera positive with the same tests

Table II

Direct IF tests performed in HSV-1-infected Vero cells with FITC conjugates of various human and animal sera

FITC conjugates of sera	IF positivity* after an incubation at			
	room temperature for 30 min with		37 °C for 3h with	
	HSV-infected cells	uninfected cells	HSV-infected cells	uninfected cells
Rabbit anti-HSV-1	++++	—	++++	—
Pig anti-influenza A	±	—	++	—
Calf anti-vaccinia	—	—	—	—
Sheep anti-human-IgG	+	—	+	—
Goat anti-human-IgG	—	—	—	—
Human anti-HSV-neg.	±	—	++	—
Normal rabbit	+	—	++	—

* Scored according to the rate of positive cells

Direct detection of Fc (IgG) receptors on HSV-infected cells. Globulins of normal mammalian sera containing no HSV antibody were conjugated with FITC and the conjugates as well as FITC conjugates of antisera to various antigens were incubated with HSV-infected and non-infected cell preparations.

The data in Table II unequivocally prove that non-anti-HSV immunoglobulins of certain animals (sheep, rabbit, pig) may bind to HSV-infected cells, thus causing false positive IF reactions. The degree of adsorption can be enhanced by raising the temperature and by prolonging the incubation period. Thus, in the indirect IF test both the human IgG and globulins of animal (sheep, rabbit or pig) origin present in the conjugate may cause false positive reactions.

The non-specific adsorptions can be eliminated, *viz.*, the former by pretreatment of the cell preparation with rabbit globulin, the latter by dilution of the conjugate in rabbit globulin solution.

The non-specific adsorption of "normal" globulins has been attributed to FC receptors present on HSV-infected living cells [4, 5]. It has been shown that only IgG molecules are adsorbed in this way. According to the present observations, the Fc receptor is functioning similarly on acetone-fixed HSV-infected cells.

Non-specific fluorescence was observed with anti-human-IgG conjugates, but not with anti-human-IgA and anti-human IgM conjugates. That the latter two themselves did not react with the Fc receptors may be explained by the low affinity to these of goat serum globulins.

The species differences in the affinity of mammalian globulins to the Fc receptors have been proved by the following experiment. Our purified

Table III

Inhibition of Fc(IgG) binding by serum globulins as tested with S. aureus protein "A" and HSV-1-infected cells

Serum globulin	Inhibition of	
	protein-"A"-induced agglutination of sensitized sheep RBC	nonspecific adsorption of human globulin to HSV-1-infected cells
Human	800*	
Rabbit	400	+
Pig	100	+
Guinea pig	50	±
Sheep	25	—
Mouse	20	—
Horse	10	—
Goat	2	—
Calf	2	—

* Reciprocals of titres against 8 haemagglutinating units

protein "A" preparation agglutinated the sensitized sheep erythrocytes due to a selective reaction of protein "A" with the Fc region of the surface-adsorbed IgG molecules. The haemagglutination reaction is inhibited to different degrees by globulins of various mammalian species *via* blocking the protein "A" molecules as Fc receptors. A similar competitive inhibition occurs when the Fc receptors of HSV-infected cells are treated with globulins of certain animal species. In an experiment of this type (Table III), the degree of inhibition was measured by means of an anti-human-IgG FITC conjugate.

It is clear that the Fc (IgG) binding was blocked to a variable degree by serum globulins of different mammalian species. The degree of inhibition elicited a close correlation when it was measured in parallel on sensitized sheep erythrocytes and HSV-infected cells as Fc receptors. High affinity to the Fc receptors was displayed by the human, rabbit and pig globulins, whereas very low, if any, affinity by the mouse, horse, goat and calf globulins.

The IgG molecules bound specifically, i. e., by their Fab region, to the virus-specific antigen of the HSV-infected cell can be examined simply by using protein "A" FITC conjugated SpA-FITC instead on the anti-human-IgG conjugate. The immunoglobulins bound by their Fc region, i. e., non-specifically are not indicated by the latter.

Although the test with the SpA-FITC reagent is about four times less sensitive in the measurement of the human antiviral IgG than that with the anti-human-IgG FITC reagent, a similarly low sensitivity would be satisfactory for detecting the IgG non-specifically bound to the HSV-infected cells. The

Table IV

IF antibody titres of human and rabbit sera tested with anti-human-IgG-FITC and SpA-FITC conjugates in HSV-infected cell preparations. Effect of pretreatment with heterologous "normal" globulin

Serum	IF titre with conjugate			
	anti-IgG-FITC		SpA-FITC	
	without	after	without	after
	pretreatment		pretreatment	
Human HSV-negative	80	<10	negative*	negative
Human HSV-negative	160	<10	negative	negative
Human HSV-positive	640	160	40	40
Human HSV-positive	640	80	20	20
Rabbit HSV-negative	320	<10	negative	negative
Rabbit HSV-positive	10,240	2560	320	320

* Negative in undiluted serum

fact that we failed to detect any non-specifically-bound IgG by SpA-FITC proves that the non-virus-specific IgG on the HSV-infected cells is bound *via* the Fc region, which is therefore inaccessible for the SpA-FITC (Table IV).

Type-specificity of HSV serological reactions. The value of serological tests including the indirect IF IgG reaction, in the diagnostics of HSV infections is limited by cross reactions between the two types of the virus. It has been reported [14] that in human primary HSV-1 infections the IgM response is type-specific, whereas the IgM antibodies produced in HSV-2 infections cross-react with HSV-1. We examined several serum samples and paired sera from cases suspect of HSV-1 or HSV-2 infections with both the anti-human IgG FITC conjugate and the SpA-FITC (Table V).

Table V

Type-specific human IgG antibodies to HSV-1 and HSV-2 as determined with anti-human-IgG FITC and SpA-FITC

Designation of serum	Titre determined with			
	anti-human-IgG FITC		SpA-FITC	
	HSV-1	HSV-2	HSV-1	HSV-2
O. K. 1/6/76	80	20	10	negative*
O. K. 22/6/76	160	40	40	negative
T. P. 4/6/76	80	40	10	5
T. P. 18/6/76	80	160	5	20
C. I. 6/2/76	40	20	10	negative
C. L. 2/3/76	160	40	80	5

* Negative in undiluted serum

The results were suggestive of the higher type-specificity of the SpA-FITC reaction.

Examination of the HSV-specific antigens and the Fc receptors on living virus-infected cells. The virus-specific antigens on the surface of unfixed cells are usually examined by the membrane-IF technique [15]. The rosette-formation technique developed by us proved also suitable for this purpose.

We incubated suspensions of HSV-infected and non-infected cells (10 000 cells/ml) with serial dilutions of HSV-positive and HSV-negative human sera in Widal tubes for 1 hr. The cells were then washed three times with PBS containing egg albumin. The suspensions were centrifuged at 1500 rpm for 5 min. Subsequently, St-FITC reagent containing 10^8 /ml staphylococcal cells was dropped into each tube. The tubes were incubated at 4°C for 16 hr, then Evans blue diluted to 1 : 5000 in 50% neutral glycerol was dropped into each tube. Several minutes later, small samples were placed onto slides and examined for IF.

Table VI shows the results of such an experiment. It is clear that the membrane IF test, similarly as the anti-HSV IgG IF tests, is less type-specific than the St-FITC rosette reaction, which is especially valuable in the exclusion of cross reactions with HSV-2 in HSV-1 infections.

A typical picture of the St-FITC rosette is shown in Fig. 1.

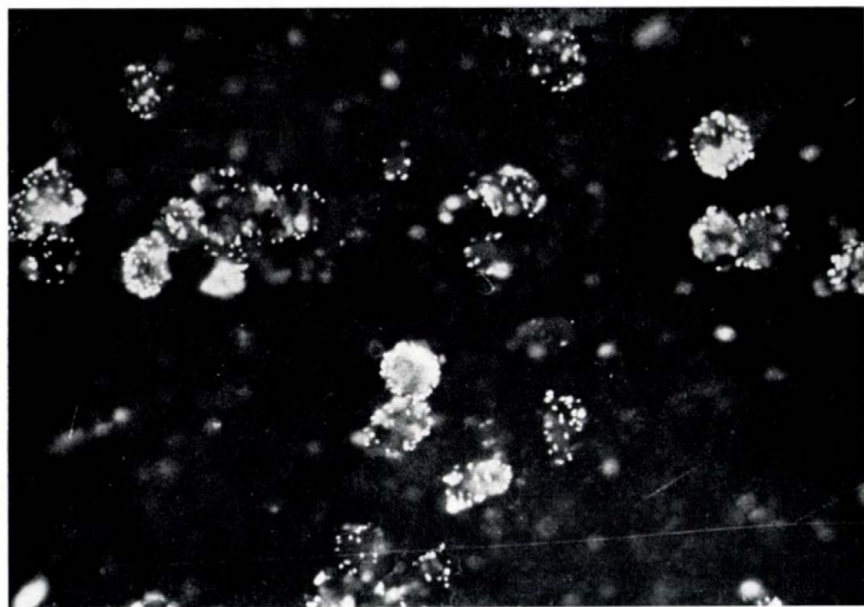


Fig. 1. Staphylococcus rosette test. HSV infected Vero cells were incubated first with human anti-HSV serum and then with FITC-conjugated Staphylococcus cells

Table VI

Anti-HSV-1 and anti-HSV-2 IgG titres of anti-HSV-positive human sera as tested by membrane IF and St-FITC rosette formation

Human sera Serial No.	Membrane-IF titres		St-FITC rosette titres	
	HSV-1	HSV-2	HSV-1	HSV-2
92	160	80	320	80
167	320	80	320	320
118	160	40	320	<10
212	160	40	160	<10
304	320	80	640	<10

Discussion

Fc receptor activity, previously described for HSV-infected living cells, has been demonstrated on acetone-fixed cell preparations. The Fc receptor is responsible for the adsorption of non-specific IgG, which considerably disturbs the determination by indirect IF of anti-HSV antibodies in human sera. The disturbing effect can be eliminated by pretreatment of the cell preparations with normal rabbit serum containing no antibodies to HSV. The blocking of the non-specific adsorption by rabbit globulin is based on a competition for the receptor sites.

The affinity of mammalian serum globulins for the receptor varies with the species. Human and rabbit IgGs have the highest affinity, the affinity of the guinea pig and pig globulins is lower and the mouse, sheep, goat, horse and calf serum globulins show very low, if any, affinity to the receptor. The agglutination of sensitized sheep erythrocytes with the protein "A" of *S. aureus* is inhibited by the serum globulins of different mammalian species in a similar degree and by a similar mechanism as that inhibiting the receptor activity of HSV-infected cells.

We have succeeded in detecting the non-specific adsorption of anti-human-IgG conjugates, but failed to demonstrate a similar adsorption of anti-human-IgA and anti-human-IgM conjugates. Since the antibodies in the conjugates were of goat origin, i. e., low-affinity antibodies, our results suggest that, similarly as the Fc receptors of the living HSV-infected cells, those on acetone-fixed cells react with IgG selectively.

The FITC conjugate of the isolated protein "A" can be used as an IF reagent for detection of the free Fc ends of IgG molecules specifically bound to viral antigens. Its use is especially favourable in the case of human, rabbit and

pig sera. It has the advantage that the reaction is not disturbed by any background fluorescence and it does not indicate the IgG molecules that are bound by their Fc region. It is no disadvantage that the reaction with the SpA-FITC is less sensitive than that with the anti-human-IgG FITC conjugate, because in the absence of non-specific binding even undiluted sera can be tested by the former method. Furthermore, the use of the SpA-FITC method on HSV preparations requires no pretreatment to eliminate false positive reactions. The false positive reactions with antihuman-IgG FITC conjugate are due to the capacity of this conjugate to react with several antigenic determinants of the IgG molecule. Hence, both the specifically and non-specifically bound IgG molecules are indicated by the reagent.

The type specificity of the widely used HSV antibody assays is poor. Determination by tests in paired sera of the type of the HSV causing a given infection needs calculation of indices [16], or cross absorption with virus-infected cell suspensions before testing [17].

We have found that the type differentiation is much more reliable and much easier with the SpA-FITC reagent than with other methods. The advantages of the protein "A" reaction also can be utilized in the staphylococcus rosette-formation test evolved by us.

The Fc (IgG) receptors appearing in herpesvirus-infected cells may have some role in the pathological process. The IgG molecules fixed by their Fc end may render the infected cell inaccessible for cytotoxic antibodies and T. cells. The fixation of complement may also be disturbed because its binding site on the IgG molecule is near the Fc region.

Fc (IgG) receptors have been detected on HSV-transformed cells [18], on Burkitt lymphoma cells [19], and IgG receptors on various human tumour cells [20, 21].

Although the role of the IgG-binding Fc receptors is still unclear, the receptors are known to be covered by immune complexes, which may block the tumour destruction due to the cell-mediated immune response [22].

REFERENCES

1. WATKINS, J. F.: *Nature (Lond.)* **202**, 1364 (1964).
2. WATKINS, J. F.: *Virology* **26**, 746 (1965).
3. SHIMIZU, Y.: *Arch. ges. Virusforsch.* **33**, 338 (1971).
4. FURUKAWA, T., HORNBERGER, E., SAKUMA, S., PLOTKIN, S. A.: *J. clin. Microbiol.* **2**, 332 (1975).
5. WESTMORELAND, D., WATKINS, J. F.: *J. gen. Virol.* **24**, 167 (1974).
6. LAUSCH, R. N., MURASKO, D. M., ALBRECHT, T., RAPP, F.: *J. Immunol.* **112**, 1630 (1974).
7. RAHMAN, A., TESCHNER, M., SETHI, K. K., BRANDIS, H.: *J. Immunol.* **117**, 253 (1976).
8. RUZICKSA, P.: *Acta morph. Acad. Sci. hung.* **12**, 275 (1964).
9. TAKÁTSY, GY.: *Acta microbiol. Acad. Sci. hung.* **3**, 191 (1955).
10. FROSGREN, A., SJÖQUIST, J.: *J. Immunol.* **97**, 822 (1966).
11. KRONVALL, G., WILLIAMS, R. C.: *J. Immunol.* **103**, 823 (1969).
12. KRONVALL, G.: *J. med. Microbiol.* **6**, 187 (1973).

13. AVRAMEAS, S., TERNYCK, T.: *Immunochemistry* **6**, 53 (1969).
14. SCHMIDT, N. J., FORGHANI, B., LENNETTE, E. H.: *Infect. and Immun.* **12**, 728 (1975).
15. NAHMAS, A. J., DEL BUONO, I., SCHNEEWEIS, K. F., GORDON, D. S., THIES, D.: *Proc. Soc. exp. Biol. (N. Y.)* **138**, 21 (1971).
16. RAWLS, W. E., IWAMOTO, K., ADAM, E., MELNICK, J. L.: *J. Immunol.* **104**, 599 (1970).
17. FORGHANI, B., SCHMIDT, N. J., LENNETTE, E. H.: *J. clin. Microbiol.* **2**, 410 (1975).
18. WESTMORELAND, D., WATKINS, J. F., RAPP, F.: *J. gen. Virol.* **25**, 167 (1974).
19. KUMAGAI, K., MINODA, J.: *Bact. Proc.* p. 181 (1970).
20. KERBEL, R. S., DAWIES, A. J. S.: *Cell* **3**, 105 (1974).
21. TÖNDER, O., KRISHNAN, E. C., JEWELL, W. R., MORSE, A. P., HUMPREY, L. J.: *Acta path. microbiol. scand. Sect. C* **84**, 105 (1976).
22. LEHNER, T., WILTON, J. M. A., SHILLITOE, E. J.: *Lancet* **2**, 60 (1975).

Address of the author:

MIKLÓS SIMON
National Institute of Hygiene
H-1966 Budapest, P. O. B. 64, Hungary

SIMPLE ION EXCHANGE BATCH TECHNIQUE FOR DETECTION OF IgM ANTIBODIES AGAINST RUBELLA AND FLAVIVIRUS ANTIGENS

G. NAGY, ILONA MEZEY and ERZSÉBET MOLNÁR

National Institute of Hygiene, Budapest

(Received January 11, 1977)

A simple ion exchange batch separation method using QAE-Sephadex A-25 gel was elaborated for separation of IgM antibodies from human sera and its applicability has been compared to that of ion exchange column chromatography for detection of IgM antibodies against rubella and tick-borne encephalitis virus antigens. Haemagglutination inhibition (HI) titrations of IgM fractions obtained by both methods were performed in 44 serum samples containing HI antibodies against rubella virus antigen and in case of 72 serum samples containing antibodies against tick-borne encephalitis virus antigen. The batch method proved to be less sensitive, and therefore less suitable than chromatography for the detection of specific IgM antibodies present in low titre several months after infection. In the case of acute or convalescent phase sera the applicability of batch technique was, however, practically identical with that of column chromatography.

Separation of IgM antibodies of human serum by ion exchange column chromatography and its use for the demonstration of rubella-specific IgM antibodies have been described in a previous paper [1]. The method proved also suitable for the demonstration of flavivirus-specific IgM antibodies occurring after tick-borne encephalitis (TBE) virus infection. Column chromatography is, however, a relatively laborious and time-consuming method needing some special equipments (e. g. absorptiometer) which hinder its use in routine diagnostic virology. Thus, elaboration of a more simple method for the separation of IgM antibodies seemed desirable. The present paper gives an account on the development of an ion exchange batch separation technique and its use for the detection of IgM antibodies to rubella and tick-borne encephalitis virus.

Materials and methods

Test sera. (a) Forty-four serum samples containing rubella haemagglutination inhibiting (HI) antibodies taken from 41 subjects were selected for rubella-specific IgM antibody tests. Twenty-six of the 41 subjects had a recent rubella-like illness, whereas 15 had contact with suspected cases of rubella.

(b) Seventy-two serum samples containing antibodies against haemagglutinating antigen of TBE virus were selected for the demonstration of flavivirus-specific IgM antibodies. Fifty-five sera were taken from 32 patients with meningitis or encephalitis in the acute or convalescent phase of the illness, whereas 17 sera from individuals with a history of serologically proven tick-borne encephalitis virus infection 9–15 months before sampling.

Sera for rubella examination were stored at -20°C without previous heat inactivation. Serum samples for TBE virus tests were subjected to heat inactivation at 56°C for 30 min before storage at $+4^{\circ}\text{C}$.

Elimination of non-specific serum inhibitors and agglutinins. For preliminary treatment before ion exchange column chromatography, 0.4 ml of serum was subjected to heparin-manganous chloride treatment and to adsorption with pigeon erythrocytes for rubella test or to that with goose erythrocytes for TBE virus tests. Details of the procedure have been described elsewhere [1].

Before batch separation, preliminary treatment of 0.1 ml serum with heparin-manganous chloride and with the above-mentioned erythrocytes was performed according to the description published in the technical guide of the Center for Disease Control [2].

Ion exchange chromatography. Pretreated serum desalted by a quick gel filtration procedure on Sephadex G-25 bed, was submitted to two-step ion exchange column chromatography in a QAE-Sephadex A-25 bed. For elution of the first protein peak containing the IgG, 0.1 M Tris-HCl buffer pH 8.1 was applied; whereas the second, IgM containing protein peak could be obtained by 0.3 M Tris-HCl buffer pH 6.6. Details concerning chromatography have been described in a previous paper [1].

Batch separation. A certain quantity of QAE-Sephadex A-25 gel was washed several times with an excess of 0.03 M Tris-HCl buffer pH 8.8. A thick slurry of gel was pipetted into previously calibrated 10×1.1 cm glass tubes until the settled volume was just above the 1.0 ml mark. Removal of the slight excess of buffer remaining above the gel proved to be unnecessary.

The 0.8 ml pretreated serum (corresponding to 0.1 ml original serum volume) was measured to the tube containing the gel. The stoppered tube was rotated end-to-end for 30 minutes to ensure complete adsorption of IgM to the gel. Unattached IgG was washed out from the gel in two subsequent steps: (1) 4-5 ml of 0.05 M Tris-HCl buffer pH 8.1 was added to the tube and mixed for 1 min and then the tube was centrifuged at 1000 rpm for 1 min and the supernatant was discarded; (b) the procedure was repeated by using 0.12 M Tris-HCl buffer pH 8.1.

In order to elute IgM from the gel, 0.1 ml volume of 2.0 M Tris-HCl buffer pH 6.6 was added to the gel and thoroughly mixed. Eluate was obtained by pressing the slurry through a linen filter retaining the gel particles.

Haemagglutination inhibition (HI) tests. Investigation of rubella HI antibody titre of the fraction obtained in batch separation was conducted as described by the Center for Disease Control [2].

Tick-borne encephalitis virus HI antibody tests were performed according to the technique described by CLARKE and CASALS [3].

Batch separation of ABO blood group typing sera. In order to determine the actual dilution of IgM antibodies obtained by the batch technique, model experiments were carried out with human ABO blood group typing "Serotyp" sera (Institute for Serobacteriological Production and Research Human, Budapest). Isohaemagglutinin titration of the IgM fraction was performed by TAKÁTSY's micro method against the corresponding group of human red blood cells. Based on the results of these experiments the batch technique resulted in eight to sixteen-fold dilution of IgM antibodies.

Immunodiffusion. Determination of IgG, IgA, and IgM antibodies in the final fraction obtained in batch separation was performed by immunodiffusion. Details of the method have been described previously [1]. Immunodiffusion experiments have shown that besides IgM only a slight trailing of IgG and IgA was present in the fraction.

2-Mercaptoethanol (2-ME) treatment. Details of 2-ME treatment of the IgM fractions obtained in ion exchange column chromatography have been described previously [1].

For 2-ME treatment of the IgM fractions obtained with the batch method, 0.025 ml volumes of the fraction were measured into two parallel wells of a "V" bottom microtitration plate; 0.025 ml of 0.15 M 2-ME freshly prepared in phosphate buffered saline pH 7.5, was added into one well, whereas in the other the same volume of saline was pipetted. The plate was shaken thoroughly, then it was covered with another plate to avoid evaporation and incubated at 37°C for one hour. Subsequently twofold dilution series of the incubated materials were prepared in appropriate buffers by 0.025 ml microdiluter. Subsequent procedures were performed according to the conventional rubella or tick-borne encephalitis virus HI tests.

Only those titres were considered to represent specific IgM antibodies which had shown an at least fourfold decrease after 2-ME treatment.

Results

Examination of sera containing rubella HI antibodies (Table I). Rubella-specific IgM antibodies were demonstrated in 15 of the 44 serum samples subjected to ion exchange column chromatography. In 11 of the 15 serum samples rubella-specific IgM antibodies could be detected by the batch method, whereas all the other sera tested following batch separation were found negative.

Examination of sera containing tick-borne encephalitis virus HI antibodies. Fifty-five acute or convalescent phase serum samples of 32 patients with suspected tick-borne encephalitis virus infection were tested for the presence of flavivirus-specific IgM antibodies both by ion exchange column chromatography and by batch separation. Specific IgM antibodies could be found in 35 sera and could not be detected in 10 sera by either method, whereas demonstration of specific IgM antibodies was successful in the case of 5 sera by column chromatography, and in the case of another 5 sera by batch separation alone (Table II). The latter 5 sera showed 2-ME resistant HI titres in the IgM fractions of column chromatography.

Table I

Rubella-specific IgM antibodies in sera of subjects with rubella-like illness or with exposition to rubella infection, by batch method and column chromatography

Batch method	Column chromatography		Total
	IgM positive sera	IgM negative sera	
IgM positive sera	11	—	11
IgM negative sera	4	29	33
Total	15	29	44

Table II

Flavivirus-specific IgM antibodies in acute or convalescent phase sera of patients with suspected tick-borne encephalitis virus infection, by batch method and column chromatography

Batch method	Column chromatography		Total
	IgM positive sera	IgM negative sera	
IgM positive sera	35	5	40
IgM negative sera	5	10	15
Total	40	15	55

Flavivirus-specific IgM antibodies were demonstrated by ion exchange column chromatography in six of the 17 serum samples taken from individuals with a history of tick-borne encephalitis virus infection 9–15 months before blood sample collection. Two of these sera were positive for specific IgM antibodies by batch separation (Table III).

Table III

Flavivirus-specific IgM antibodies in sera of patients taken 9–15 months after tick-borne encephalitis virus infection, by batch technique and column chromatography

Batch method	Column chromatography		Total
	IgM positive sera	IgM negative sera	
IgM positive sera	1	1	2
IgM negative sera	5	10	15
Total	6	11	17

Discussion

The main three immunoglobulin classes bind differently to ion exchangers due to differences in their isoelectric points [4]. This offers various possibilities for their separation from human serum. A method for purification of IgG by a simple batch procedure using anion exchanger gel was reported by BAUMSTARK *et al.* [5]. This method and its modification by WEBB [6] were successfully applied by several authors [7–11]. Though the procedure offered a possibility for purification of IgM from human serum, the IgM rich fractions obtained with WEBB's method by HORNSLETH *et al.* [10, 11] contained also a significant quantity of IgG. By the method reported in the present paper, an IgM rich fraction with almost entirely reduced IgG content could be obtained allowing direct titration of specific IgM antibodies by the HI test.

In the case of acute or convalescent phase sera, ion exchange column chromatography proved to be only slightly more efficient in demonstrating rubella and flavivirus-specific IgM antibodies than the batch method. Examination of sera taken 9–15 months after the tick-borne encephalitis virus infection showed that ion exchange column chromatography was certainly more sensitive. The difference in sensitivity was caused by two factors, *viz.* (a) as it was demonstrated in model experiments with blood group typing sera, IgM antibodies became about two times more diluted in fractions of batch separation than in fractions obtained in column chromatography; (b) the difference in

dilution was further increased by the technique applied for the 2-ME treatment of batch fractions.

In spite of its lower sensitivity, the batch technique has several advantages over column chromatography. It is a simple method without any need for special equipments, requires only 0.1 ml serum, and it is suitable for the rapid simultaneous fractionation of a considerable number of serum samples. On the basis of these facts, ion exchange batch separation may help to clear up virus infections when antibody titrations of paired sera are not informative. The main advantage of the method is that it shortens the time needed for laboratory diagnosis by detection of the infection by testing a single serum sample in the early stage of the illness.

Acknowledgements. We are indebted to Dr. I. DÖMÖK for helpful criticism during preparation of the manuscript. Thanks are also due to Mrs. É. GÁSPÁR, Mrs. Zs. PALÁNSZKY and Mrs. É. HETÉNYI for excellent technical assistance.

REFERENCES

1. NAGY, G., MEZEY, I.: *Acta microbiol. Acad. Sci. hung.* **24**, 111 (1977).
2. A Standardized rubella HAI test. Immunology Series No. 2. Procedural Guide. Center for Disease Control, Atlanta, Georgia 1970.
3. CLARKE, D. H., CASALS, J.: *Amer. J. trop. Med. Hyg.* **7**, 561 (1958).
4. Бурлев, В. А., Голошова, Т. В., Кириухин, И. Ф.: *ж. микробиол.* **3**, 96 (1976)
5. BAUMSTARK, J. S., LAFFIN, R. J., BARDAWIL, W. A.: *Arch. Biochem. Biophys.* **108**, 514 (1964).
6. WEBB, A. J.: *Vox Sang. (Basel)* **23**, 279 (1972).
7. ALTEMEIER, W. A. III., MUNDON, F. K., TOP, F. H. JR., RUSSEL, P. K.: *Appl. Microbiol.* **19**, 785 (1970).
8. GRAHAM, H., MORRISON, M., CASEY, E.: *Vox Sang. (Basel)* **27**, 363 (1974).
9. GRAHAM, H., MORRISON, M., MACANDREW, R.: *Vox Sang. (Basel)* **29**, 371 (1975).
10. HORNSLETH, A., LEERHØY, J., GRAUBALLE, P., SPANGGAARD, H.: *Acta path. microbiol. scand. Sect. B.* **82**, 742 (1974).
11. HORNSLETH, A., LEERHØY, J., GRAUBALLE, P., SPANGGAARD, H.: *Infect. and Immun.* **11**, 804 (1975).

Address of the authors:

GÁBOR NAGY, ILONA MEZEY, ERZSÉBET MOLNÁR
National Institute of Hygiene
H-1966 Budapest, P. O. B. 64, Hungary

QUANTITATIVE ANALYSIS OF MC29 VIRUS IN PLASMA OF HEPATOMA-BEARING TURKEY POULTS BY DETERMINATION OF REVERSE TRANSCRIPTASE ACTIVITY AND RADIOIMMUNO-ASSAY

MARGARITA TÁLAS, L. G. ZAKHAROVA, IVANA STÖGER, A. D. ALTSTEIN and E. G. PIKER

Microbiological Research Group of the Hungarian Academy of Sciences, Budapest and D. I. Ivanovsky Institute of Virology, U. S. S. R. Academy of Medical Sciences, Moscow

(Received January 13, 1977)

The optimal conditions for measuring RNA-directed DNA polymerase activity of MC29 virus using poly (rA) . oligo (dT) as template were determined. A radioimmuno-assay using group specific proteins of AMV was also elaborated to detect MC29 virus. Radioimmuno-assay proved to be a much more sensitive method for measuring amounts of MC29 virus than the determination of reverse transcriptase activity.

Myelocytomatosis virus 29 (MC29) isolated in Bulgaria from a Rhode Island Red chicken with spontaneous myelocytomatosis [1] is a suitable model for the study of several questions of viral cancerogenesis. Our observations showed (unpublished data) that not every strain of MC29 induced transformation in chick embryo cell cultures, thus we had no reliable *in vitro* method for the quantitative analysis of virus preparations. Determination of the lethal or tumorigenic dose of the virus *in vivo* is inconvenient and expensive.

RNA-directed DNA polymerase activity was detected in the virions of different leukoviruses including MC29 [2]. It was shown that the reverse transcriptase activity assay may be utilized for the quantitative analysis of murine [3–5] and pig [6] oncornaviruses.

In the present experiments we have studied the possibility of quantitative determination of MC29 virus by reverse transcriptase test and the results were compared with those obtained by radioimmuno-assay of specific viral proteins.

Materials and methods

Viruses. One day old turkey poults were infected intravenously with MC29 virus (10^5 LD₅₀ per 0.2 ml) and plasma from birds with hepatomas [7] was collected on the 12th day after infection. The plasma was centrifuged at 15 000 g for 30 min and then virus particles were pelleted by centrifuging at 100 000 g for 90 min. The pellet was resuspended in a buffer containing 0.01 M Tris HCl (pH 7.5), 0.1 M NaCl and 0.001 M EDTA (TSE buffer) to obtain a 6-fold concentration of the original volume of plasma, and it was stored at 4°C. For the reverse transcriptase test, virus preparations were diluted with TKD buffer (0.05 M Tris HCl, pH 7.8 0.06 M KCl and 0.002 M dithiotreitol).

MC-29 virus was kindly supplied by Prof. Z. MLADENOV, Institute of General and Comparative Pathology, Sofia, Bulgaria. Avian myeloblastosis virus (AMV) was obtained from Dr. V. M. ZHDANOV's laboratory (Institute of Virology, Moscow).

Reverse transcriptase test. The standard reaction mixture contained (in 0.1 ml): TKD buffer, 0.00025 M MnCl_2 , 0.05% (w/v) triton X-100, 0.5 μg thymidine triphosphate, 1 μCi [^3H]thymidine triphosphate (Amersham) and 0.02 OD₂₆₀ exogenous primer template poly (rA). oligo (dT). MC29 virus preparation described above was added (0.05 ml) to the reaction mixture (0.05 ml) and incubated at 37°C for 1 hr. The reaction was stopped by the addition of 0.5 ml distilled water, 0.1 ml yeast RNA (2 mg per ml) and 0.3 ml of a 2 : 1 mixture of 50% TCA and saturated sodium pyrophosphate solution. Samples were sedimented on Millipore filters and washed with 5% TCA and ethanol. Radioactivity on the filters was determined in a scintillation counter (Packard, TriCarb). If the addition of exogenous template resulted in an at least 4-fold increase of the radioactivity as compared to the control, reverse transcriptase activity was considered positive.

Radioimmuno-assay (RIA). AMV proteins were separated on Sephadex G-100 equilibrated with Tris buffer containing 0.1% 2-mercaptoethanol, 0.3 M NaCl and 0.2% triton X-100. The mixture of group-specific proteins p 19 and p 27 (2.5 μg) was radioiodinated (1 mCi of ^{125}I) the chloramine T method [8], using 30 μg /0.15 ml of chloramine T. The labelled proteins were separated from free ^{125}I on Sephadex G-25. Various dilutions of rabbit anti-AMV immune serum [9] were added to the labelled proteins and the dilution giving 30–50% fixation was used in RIA.

MC29 virus preparation (see "viruses") was frozen and thawed and then diluted in a buffer containing 0.02 M Tris HCl (pH 7.4), 0.1 M NaCl, 0.001 M EDTA and 0.2% (w/v) triton X-100. Samples of several dilutions of MC29 virus in 0.1 ml were mixed with 0.01 ml of diluted anti-AMV serum and radiolabelled AMV proteins. After incubation at 37°C for 2 hr, goat anti-rabbit globulin (0.1 ml) was added and the reaction mixture was incubated for 1 hr at 37°C and then for 18 hr at 4°C. After centrifuging at 2000 rpm the radioactivity of the pellet was counted in a gamma counter (Packard).

Results

Figure 1 shows the reverse transcriptase activity of MC29 virus at various incubation temperatures (0°, 20° and 37°C) in the function of incubation time. Maximum activity was achieved by incubation at 20°C for 180 min or at 37°C for 120 min. Incubation at 0°C proved also suitable but the enzyme activity was much lower than at higher incubation temperatures.

The effect of various concentrations of bivalent ions (Mg^{2+} and Mn^{2+}) on the enzyme activity is shown in Fig. 2. It can be seen that 6 mM of MgCl_2 or 0.5 mM of MnCl_2 provide optimum conditions for the determination of reverse transcriptase activity.

The effect of triton X-100 on RNA-directed DNA polymerase activity was also examined in the presence of Mn^{2+} ions at 37°C. Maximum activities were obtained after adding of 0.025–0.2% triton X-100 to the reaction mixture (Fig. 3).

DNA-polymerase activity of our virus preparation was also measured, using two different exogenous templates: poly (rA). oligo (dT) and poly (dA). oligo (dT). Table I shows that enzyme activity was much higher using poly (rA). oligo (dT) as a template, indicating clearly the viral origin of the enzyme.

After standardization of conditions influencing MC29 virus reverse transcriptase assay (concentration of bivalent ions and triton X-100, incubation temperature, exogenous template) the relation between plasma dilutions

Table I

Reverse transcriptase activity of MC29 virus tested in the presence of two different exogenous templates

Reverse transcriptase activity, per cent*			
Mn ²⁺		Mg ²⁺	
poly (rA) . oligo (dT)	poly (dA) . oligo (dT)	poly (rA) . oligo (dT)	poly (dA) . oligo (dT)
100	4	91	17

* As compared to that with poly (rA) . oligo(dT) in the presence of Mn²⁺ ions

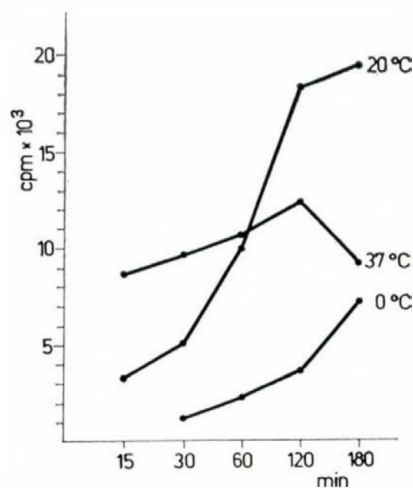


Fig. 1. Reverse transcriptase activity of MC29 virus, at various incubation temperatures in function of time. The standard reaction mixture was used (see "Materials and methods")

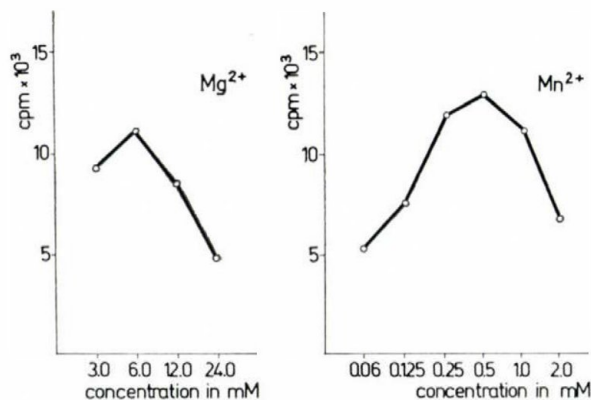


Fig. 2. Effect of concentration of Mg²⁺ and Mn²⁺ on MC29 virus polymerase activity

and RNA-dependent DNA polymerase activity was tested. Figure 4 shows the enzyme activity as a function of plasma dilutions. A direct relation can be seen.

Figure 5 shows the results of measuring of MC29 virus preparation by RIA. It is clear that a mixture of labelled p 19 and p 27 of AMV can be utilized for determination of MC29 virus as using AMV and MC29 viruses the two inhibition curves were parallel. Knowing the number of virions in the AMV

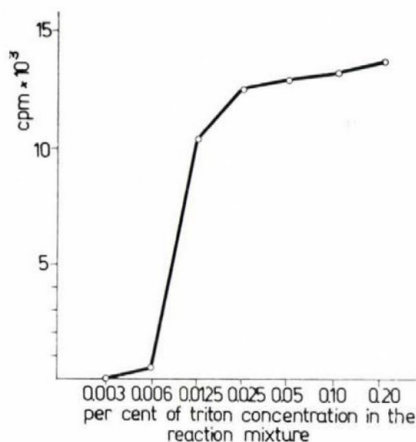


Fig. 3. Effect of triton X-100 concentration on MC29 virus polymerase activity

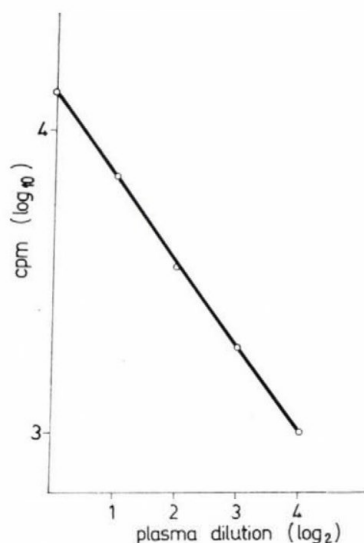


Fig. 4. Relation of plasma dilutions of turkey poult bearing primary MC29 hepatomas and reverse transcriptase activity

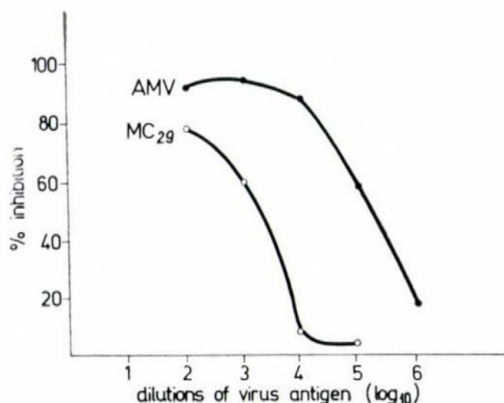


Fig. 5. Titration of MC29 virus by RIA using AMV as a standard. MC29 virus containing plasma of turkey poult and an AMV pool ($10^{12.5}$ virions per ml) were tested by RIA with ^{125}I labelled AMV protein mixture (p 19 and p 27) and rabbit anti AMV immune serum. The parallelism of the two inhibition curves allows to estimate the number of virions in MC29 virus preparation

pool used ($10^{12.5}$ virions per ml), the number of MC29 virions in the plasma preparations could be calculated.

Data referring to plasma of turkey poult bearing primary MC29 hepatomas are summarized in Table II.

Table II

Plasma of turkey poult bearing MC29 virus-induced hepatomas

Infectivity* LD ₅₀ /ml	50% tumour inducing dose/ml*	Reverse transcriptase activity** cpm/ml	Virions per ml determined by RIA
$10^{5.5}$	$10^{1.8}$	2×10^5	$10^{9.7}$

* In vivo titration in newly hatched turkey poult

** In standard test with exogenous template poly (rA) . oligo (dT)

Discussion

RNA-directed DNA polymerase activity of oncogenic RNA viruses was determined under different experimental conditions [10]. As it was shown [11], a minor change of the optimum requirements resulted in a sharp decrease of enzyme activity.

SCOLNICK *et al.* [12] have shown that the preference of viral polymerase for bivalent ions (Mg^{2+} or Mn^{2+}) depends on the template used. We have observed no differences in polymerase activity of MC29 virus in the presence of 6 mM Mg^{2+} or 0.5 mM Mn^{2+} ions applying the synthetic exogenous template poly

(rA). oligo (dT). The enzyme activity was more sensitive to changes of Mg^{2+} than to those of Mn^{2+} . Polymerase activity did not change noticeably on a two-fold increase or decrease of Mn^{2+} .

As it was shown by WATERS and YANG [11, 13], the optimum temperature for polymerase activity depends on the thermal stability of the template primer and the source of enzyme. Polymerase activity of MC29 virus could be detected at 0°, 20° and 37°C, but at lower temperatures a longer incubation was required to attain the reaction optimum.

Viral and cellular DNA polymerase activities could be distinguished by using two synthetic oligohomopolymer templates, such as poly (rA) . oligo (dT) and poly (dA) . oligo (dT) [14]. The preference of polymerase of the plasma of MC29 virus infected turkey poult for poly (rA) . oligo (dT) over poly (dA) . oligo (dT) shows that the observed enzyme activity was of viral origin.

The direct relation between dilutions of plasma of the tumour-bearing turkeys and DNA-polymerase activity indicates that the determination of reverse transcriptase activity may be used for quantitative analysis of MC29 virus, if the presence of other oncornaviruses can be excluded.

Results shown in Fig. 5 indicate that for the determination of MC29 virus RIA is a more sensitive method than the testing of reverse transcriptase activity. By RIA, MC29 virus could be detected even in 10^{-4} dilutions of plasma of tumour-bearing turkeys, while undiluted or only 2–4-fold dilutions of plasma proved suitable for the determination of polymerase activity. Using a standard virus pool with a known number of virions, RIA can be utilized for the titration of MC29 virus preparations.

Acknowledgement. The authors are indebted to Prof. I. FÖLDES, Director of the Microbiological Research Group of the Hungarian Academy of Sciences, for critical reading of the manuscript.

REFERENCES

1. IVANOV, X., MLADENOV, Z., NEDYALKOV, S., TODOROV, T. G., JAKIMOV, M.: Bull. Inst. Path. comp. Anim. **10**, 5 (1964).
2. WEBER, G. H., KIESSLING, A. A., BEAUDREAU, G. S.: J. Virol. **7**, 214 (1971).
3. AARONSON, S. A., TODARO, G. J., SCOLNICK, E.: Science **174**, 157 (1971).
4. ГЕРАСИНА, С. Ф., ЖДАНОВ, В. М., АЛТШТЕИН, А. Д., ЗАХАРОВА, Л. Г., СЕВЕСТИЯНОВА, М. В., АРГИРОВА, Р. М., СМЕРНОВ, В. Д.: Молекулярная биология вирусов (Жданов, В. М.). Д. И. Ивановский Институт, Москва 1973, стр. 57.
5. SALZBERG, S., ROBIN, M. S., GREEN, M.: Virology **53**, 186 (1973).
6. ALTSTEIN, A. D., ARGIROVA, R. M., VYAZOV, S. O., GERASINA, S. F., ZHDANOV, V. M.: Acta virol. **18**, 369 (1974).
7. SCHAFF, Zs., TÁLAS, M., STÖGER, I., FÖLDES, I., LAPIS, K.: Acta microbiol. Acad. Sci. hung. **24**, 75 (1977).
8. GREENWOOD, F. C., HUNTER, W. M., GLOVER, J. S.: Biochem. J. **89**, 114 (1963).
9. SUNI, J., VAHERI, A., RUOSLAHTI, E.: Intervirology **1**, 119 (1973).
10. TEMIN, H. M., BALTIMORE, D.: Advanc. Virus Res. **17**, 129 (1972).
11. WATERS, L. C., YANG, W. K.: Cancer Res. **34**, 2585 (1974).

12. SCOLNICK, E., RANDS, E., AARONSON, S. A., TODARO, G. J.: Proc. nat. Acad. Sci. (Wash.) **67**, 1789 (1970).
13. WATERS, L. C., YANG, W. K.: Fed. Proc. **30**, 1163 (1971).
14. ROBERT, M. S., SMITH, R. C., GALLO, R. C., SERIN, P. S., ABRELL, J. W.: Science **176**, 798 (1972).

Address of the authors:

MARGARITA TÁLAS, IVANA STÖGER
Microbiological Research Group of the Hungarian
Academy of Sciences,
Pihenő út 1, H-1529 Budapest, Hungary

L. G. ZAKHAROVA, A. D. ALTSTEIN, E. G. PIKER
D. I. Ivanovsky Institute of Virology, U. S. S. R.
Academy of Medical Sciences
123098 Moscow, U. S. S. R.

IMMUNOLOGICAL DEMONSTRATION OF ONCORNAVIRUS GENOME IN LEUCOCYTES IN ACUTE AND CHRONIC MYELOGENOUS LEUKAEMIA AND PRELEUKAEMIA

F. D. TÓTH, J. JAKÓ, L. VÁCZI, K. RÁK and I. RÉDAI

Institute of Microbiology and Second Department of Medicine, University Medical School, Debrecen

(Received January 28, 1977)

Leucocytes from 10 patients with acute myelogenous leukaemia (AML), from 16 with chronic myelogenous leukaemia (CML), from 9 with potential preleukaemia and from 42 control patients were tested by indirect immunofluorescence for the presence of cytoplasmic antigens of C-type oncornaviruses. Leucocytes from all cases of AML contained the LPV-specific p30 antigen which, depending on the stage of disease, cross-reacted with antisera to simian and/or murine cytoplasmic oncornavirus antigens. Of the 9 preleukaemia cases, 6 were LPV-p30-positive, and the majority of these cross-reacted with antisera to simian p30 antigens. Of the 11 CML patients responding well to chemotherapy, 5 were found negative, the remaining 6 had endogenous oncornavirus antigen in leucocytes. Antigens characteristic of AML were demonstrated in the leucocytes of 5 CML patients including the poor responders in the early blastic and fully developed blastic stages.

The possibility that human leukaemias may be caused by C-type oncornaviruses was raised on the basis of electron microscopic studies. DMOCHOWSKI and GREY [1] demonstrated particles resembling murine leukaemia virus virions in tissues of patients with leukaemia. Later RNAs homologous to nucleic acids of C-type oncornaviruses were demonstrated by nucleic acid hybridization [2, 3] and more recently the presence of reverse transcriptase related to that of primate leukaemia sarcoma viruses has been shown in cells from human leukaemia cases [4]. Investigations with various immunological methods have suggested that human tumours of mesodermal origin may contain C-type oncornavirus [5–7]. In recent years, several authors [8–10] have reported on the isolation of C-type viruses from patients with acute myelogenous leukaemia (AML). Among the hypothetical human leukaemia viruses, ANDZHAPARIDZE'S LPV strain may be of great importance because this strain is capable of causing leukaemia in experimentally inoculated *Macaca mulatta* monkeys [11].

In the immunological study of C-type oncornaviruses the demonstration of the p30 antigen, a polypeptide of about 30 000 molecular weight (12,13), is of the greatest interest. This antigen has two determinants, *viz.*, a group-specific (gs-1) and an interspecies-specific (gs-3) one [12, 14]. Based on these determinants, oncornaviruses can be divided into closely and less closely related groups [15]. The reverse transcriptase enzymes [16] as well as the main glycoprotein antigens (gp-70) [17] of the viral envelope in these groups show similar cross reactions as the p30 antigens.

In order to clarify the interrelations between C-type oncornaviruses and human leukaemias, the human specificity of the suspect viral isolates and antigens should be checked and oncornavirus should be demonstrated in preleukaemias.

In the present work, an attempt has been made at demonstrating C-type oncornavirus cytoplasmic antigens in peripheral leucocytes of patients suffering from AML or chronic myelogenous leukaemia (CML) or being in the stage of potential preleukaemia. Anti LPV p30 serum and antisera to the corresponding antigens of different mammalian C-type oncornaviruses were used for this purpose.

Materials and methods

Purification of leucocytes. BOYLE's [18] method was used. Fresh citrated blood was poured off. The cell sediment was resuspended in a mixture of 0.1 volume 5% EDTA solution and 8 volumes of 0.83% NH_4Cl solution. The suspension was incubated at 4°C for 10 min and centrifuged. The supernatant was poured off. To remove the residual erythrocytes from the sediment, the cells were resuspended in NH_4Cl solution and centrifuged again. The leucocytes thus obtained were washed three times with, and resuspended in, PBS of pH 7.2. The cell suspension was diluted with PBS to the desired cell count.

Antisera, fluorescent conjugates. Rabbit immune serum to detergent treated LPV was kindly supplied by Professor O. G. ANDZHAPARIDZE (Scientific Research Institute for Viral Preparations, Moscow, U. S. S. R.). Goat sera to the p30 antigens of three exogenous mammalian oncornaviruses, viz., Rauscher leukaemia virus (RLV) p30 (5S-123), feline leukaemia virus (FeLV) Theilen strain p27 (5S-534), simian sarcoma virus (SSV-1) p28 (2S-696) and those to two endogenous viruses of mammalian origin, viz., RD-114 p30 (2S-781), endogenous Baboon virus (M7 strain) p28, were received from the Huntingdon Research Laboratory (Brooklandville, Maryland, U. S. A.). The rabbit antiserum G-38 to gibbon lymphoma virus (GALV) p27 was received from Pfizer International Inc., New York. We designate the sera in the following with the prefix anti-, and the abbreviated name of the strain. In immunofluorescence studies, fluorescein-isothiocyanate-labelled goat anti-rabbit-IgG and rabbit anti-goat-IgG sera (Hyland, Costa Mesa, California, U. S. A.) were used.

Indirect cytoplasmic immunofluorescence (IF). Seven drops from each leucocyte suspension to be tested, containing 1.4×10^5 cells per drop, were dried on slides and fixed in acetone for 10 min at room temperature, then a drop of one of the antisera was added to each. The dilution of the antisera was tenfold, the dilution giving the homologous immunodiffusion titre. The slides were incubated in a wet chamber at 37°C for 30 min, washed three times with distilled water and dried at room temperature. Then, one drop of 1 : 20-diluted anti-rabbit IgG or anti-goat IgG was added to each sample. After an incubation as above the slides were washed with distilled water three times and allowed to dry; one drop containing equal volumes of glycerol and PBS was added and each preparation was covered with a coverslip. The preparations were examined under the fluorescence microscope.

Results

Two types of reaction were observed, viz., a negative reaction showing no IF, and fluorescence in 80–100% of the cells. The positive reaction is shown in Fig. 1.

The results obtained with cell samples from 10 patients suffering from myeloblastic or acute myelomonocytic leukaemia are summarized in Table I.

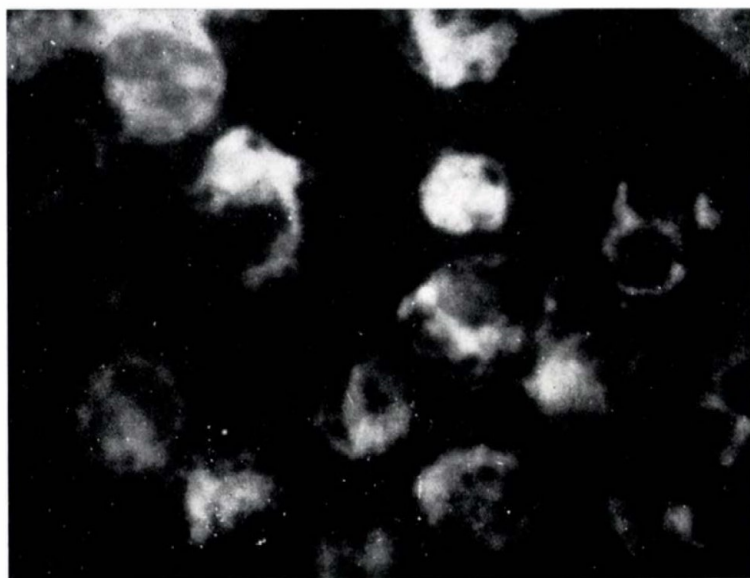


Fig. 1. Immunofluorescence with anti-LPV serum in peripheral leucocytes of a patient (V. S) suffering from acute myelogenous leukaemia

Table I

Immunofluorescence demonstrated with labelled antisera to the p30 antigens of LPV and other mammalian oncornaviruses in leucocytes of patients with acute leukaemia

Patient		Diagnosis	Leucocytes		Fluorescence with antisera to p30 of						
Initials	Age, years		cells/ μ l	blasts, per cent	LPV	GALV	SSV-1	Baboon	FeLV	RD-114	RLV
K. F.	56	AMML	118000	100	+	+	+	+	—	+	+
K. F.	30	AML*	76000	97	+	+	+	+	—	+	+
Sz. P.	46	AML	26400	91	+	+	+	+	—	+	+
H. M.	24	AML	54000	64	+	+	+	+	—	+	+
K. Zs.	72	AMML	22000	38	+	+	+	+	—	—	+
V. S.	70	AML	44000	20	+	+	—	—	—	—	—
S. B.	70	AML	13500	20	+	+	—	+	—	+	—
H. Z.	33	AML	10000	14	+	+	—	+	—	—	—
V. L.	50	AML	2 400	0	+	+	—	—	—	—	—
P. M.	16	AMML	2 600	0	+	—	—	—	—	—	—

AMML = Acute myeloblastic leukaemia

AML = Acute myelomonocytic leukaemia

At the time of sampling, the first 8 patients were in the progressive stage, whereas the last two were in clinical remission. The leucocytes of all the 10 patients proved to be positive with anti LPV serum and negative with anti FeLV serum. The leucocytes from the patients being in a highly advanced stage of leukaemia (the first five in Table I) gave the IF reaction with all the other sera except K. Zs.'s cells with the anti-RD-114 serum. In three cases being in partial remission (V. S., S. B and H. Z., having 14–20% blast forms), was no reaction with the anti-RLV serum, whereas in two cases (V. L. and P. M.) having no blast forms at the time of blood sampling only two (anti-LPV and anti-GALV) and only one serum (anti-LPV), respectively, elicited IF.

In four cases, leucocyte samples were tested with antisera in different stages of the illness (Table II).

The disappearance of blast forms was accompanied by the disappearance of the cross reaction with the anti-RLV serum while the IF with the anti-LPV serum remained unchanged. As to the reactions with the other oncornavirus antibodies, the cells of the four patients behaved differently. The leucocytes of K. Zs. lost all the cross reactions in spite of the presence of 7% blast cells.

Of nine patients with potential preleukaemia, two proved to be negative with all the seven antisera, the remaining except one (F. I.) reacted with the anti-LPV serum and 1–3 other antisera (Table III).

Table II

Immunofluorescence with antisera to p30 antigens in relation to leucocyte counts and per cent blast forms in the leucocyte populations of patients with acute leukaemia

Patient		Diagnosis*	Leucocytes		Fluorescence with antisera to p30 of						
Initials	Age, years		cells/ μ l	blasts, per cent	LPV	GALV	SSV-1	Baboon	FeLV	RD-114	RLV
K. F.	30	AML	76 000	97	+	+	+	+	—	+	+
			600	0	+	+	—	+	—	—	—
Sz. P.	46	AML	26 400	91	+	+	+	+	—	+	+
			4000	0	+	+	+	—	—	—	—
K. Zs.	72	AMML	22 000	38	+	+	+	+	—	—	+
			2800	7	+	—	—	—	—	—	—
T. Z.	45	AML	800	12	+	+	+	—	—	—	—

* See footnote, Table I

Table III

Immunofluorescence demonstrated with antisera to p30 antigens in leucocytes of patients with potential preleukaemia

Patient		Diagnosis	Fluorescence with antisera to p30 of						
Initials	Age, years		LPV	GALV	SSV-1	Baboon	FeLV	RD-114	RLV
N. P.	44	Pancytopenia	—	—	—	—	—	—	—
T. L.	61	Pancytopenia	+	+	—	+	—	+	—
F. K.	20	Pancytopenia	+	+	—	—	—	—	—
P. F.	29	Pancytopenia	+	+	+	—	—	—	—
M. I.	63	Pancytopenia	—	—	—	—	—	—	—
P. M.	25	Pancytopenia	+	—	—	—	—	—	—
K. Gy.	46	Thrombocytopenia	+	+	+	—	—	—	—
P. J.	47	Refractory anaemia	+	—	—	+	—	+	—
F. I.	59	Granulocytopenia	—	—	—	+	—	—	—

In three cases, the cells reacted with one or both of the antisera to exogenous simian oncornaviruses (GALV and SSV-1) but with neither of the anti-endogenous oncornavirus sera (Baboon and RD-114); one patients' leucocytes reacted with both of the latter sera but neither of the anti-exogenous oncornavirus sera. Leucocytes from only one (T. L.) of these patients reacted at the same time with anti-exogenous and anti-endogenous oncornavirus sera. In one case (F. I.) the IF reaction was positive with the anti-Baboon serum although the other antigens including LPV p30 could not be detected. We observed noproductive reaction with anti-FeLV and anti-RLV (antisera to non-primate exogenous oncornaviruses) in this group of patients.

The 16 patients with CML divided into three groups on the basis of the course of their illness. (Table IV); *viz.*, 11 patients could easily be kept in balance with chemotherapy (group *a*); two patients were kept in balance with difficulty (group *b*) and three patients were in the early blastic or blastic stage (group *c*).

In group *a*, five patients were found to be completely negative for oncornavirus cytoplasmic antigens, whereas endogenous oncornavirus antigen was demonstrated in the leucocytes of the remaining six (one or both of the "anti-endogenous" sera gave a positive reaction). In group *b*, the leucocytes of both patients were positive for all the four primate virus antigens, and also for the RD-114 antigen, and negative for the remaining two. In group *c*, L. M. was positive for the four antigens of primate origin and also for the RLV antigen and negative for the two of feline origin. The leucocytes from the other two patients in this group reacted with only two sera, *viz.*, anti-LPV and anti-GaLV.

Table IV

Immunofluorescence demonstrated with antisera to p30 antigens in leucocytes of patients suffering from chronic myelogenous leukaemia

Patient		Diagnosis	Leucocytes		Fluorescence with antisera to p30 of						
Ini- tials	Age, years		cells/ μ l	blasts, per cent	LPV	GALV	SSV-1	Baboon	FeLV	RD-114	RLV
P. S.	43	CGL*	56 000	3	—	—	—	+	—	+	—
B. B.	64	CGL	18 000	0	—	—	—	—	—	—	—
M. Z.	35	CGL	30 600	0	—	—	—	+	—	—	—
K. I.	80	CGL	27 300	0	—	—	—	—	—	—	—
K. I.	64	CGL	13 300	0	—	—	—	—	—	—	—
M. S.	52	CGL	22 000	1	—	—	—	+	—	—	—
B. J.	22	CGL	16 100	0	—	—	—	+	—	—	—
N. I.	55	CGL	13 500	0	—	—	—	—	—	—	—
Sz. J. ..	63	CGL	14 000	0	—	—	—	+	—	+	—
N. M.	80	CGL	25 400	0	—	—	—	+	—	+	—
E. Eg	80	CGL	45 200	1	—	—	—	—	—	—	—
T. I.	65	CGL	96 800	0	+	+	+	+	—	+	—
K. M.	52	CGL	66 400	6	+	+	+	+	—	+	—
B. S	56	CGL preblast*	50 600	12	+	+	—	—	—	—	—
L. M.	40	CGL bl. cr.**	19 000	45	+	+	+	+	—	—	+
Sz. M.	51	CGL bl. cr.**	116 000	94	+	+	—	—	—	—	—

CGL = chronic granulocytic leukaemia

* preblastic stage

** blastic crisis

Thirty-seven patients suffering from non-haematological disease and five with cytopenia qualified as non-preleukaemic served as controls. Cytoplasmic fluorescence with the anti-Baboon serum was observed in two cases. All the other leucocyte samples were negative.

Discussion

We succeeded in demonstrating the LPV p30 antigen in the leucocytes from all the 11 AML cases tested. ANDZHAPARIDZE *et al.* [19] were successful in only 17 of their 29 acute leukaemia cases. However, they did not indicate the type of leukaemia in their cases. In lymphoid tumours, if such cases were included, lymphotropic herpesviruses may have had a role.

Our positive results obtained with antisera to simian and murine p30 antigens are in accordance with the results published by ZURCHER *et al.* [7] and NOOTER *et al.* [10]. These authors observed positive reactions between anti-SSV-1 and anti-RLV serum on the one hand and human sarcoma and leukaemia cells on the other. Our negative results with the anti-FeLV serum are suggestive of the human specificity of the LPV p30 antigen, as the exogenous primate p30 antigens were found to be related to the corresponding murine oncornavirus antigen, but not to the feline one [15].

The LPV-specific p30 antigen was detectable in two AML patients in remission. The continuous presence of oncornavirus, or at least its genome, might be responsible for the exacerbations following remission. This view is supported by the biochemical investigations published by VIOLA *et al.* [20]. These authors succeeded in demonstrating complexes consisting of reverse transcriptase and virion RNA in eight of 10 patients during complete remission of acute leukaemia. The complexes were present in peripheral leucocytes of normal morphology.

Our results have shown that the cross reactions with simian and murine p30-specific antibodies are restricted during remission, supposedly due to a quantitative decrease in the LPV p30 antigen. An alternative explanation might be the disappearance of blasts, as in a cell culture developed by GALLAGHER *et al.* [21] from leucocytes of a patient with AML, C-type particles were mainly released from undifferentiated blast cells. The results in Table II seem to be inconsistent with the latter explanation, for the complete disappearance of the $> 90\%$ blast forms from the peripheral blood of two patients was not accompanied by such a restriction of the cross reaction as could have been expected on the basis of the data of two patients in remission (V. L. and P. M. in Table I). Furthermore, the spectrum of the cross reactions did not change while the percentage of blast cells in the leucocyte population of T. Z. fell from 12% to 0%. Three patients, in spite of intensive cytostatic therapy, failed to show remission. We believe that the correlation between the expression of viral antigen and the frequency of blast cells, if it exists, is of minor importance, but it seems to depend on the lack of a tendency to develop remission.

Of the nine patients with potential preleukaemia, seven proved to be positive for oncornavirus antigen; six of these showed the spectrum characteristic of acute leukaemia. We suppose that the presence or first appearance of exogenous oncornavirus antigens may be associated with an increased probability of malignant transformation. The fact that P. M. developed erythroleukaemia eight weeks after the last preleukaemia finding is in accordance with this view. VOŠKA *et al.* [22] found a positive correlation between the detectability of C-type oncornavirus and its reverse transcriptase on the one hand and the subsequent appearance of leukaemia on the other.

It is not surprising that in the blastic stage of CML, leucocytes were

found positive for LPV p30 as well as for the corresponding simian and murine antigens. In these cases the CML had developed into a condition resembling acute leukaemia. The antigen profile of the preblastic and early blastic stages is in accordance with this metamorphosis.

The difference in antigen expression between CML cases responding well and those resistant to chemotherapy is also of interest. Both patients (T. I. and K. M.) in the poorly responding group were positive for LPV p30 as well as for the exogenous simian antigens and the two endogenous antigens. In the well-responding group, five of the 11 patients were found negative and the remaining six reacted only with antisera to endogenous oncornaviruses. The sensitivity or resistance to chemotherapy may be of prognostic importance as in the case of K. M., who developed fatal blastic leukaemia 10 weeks after the last testing.

Endogenous oncornavirus antigen was also demonstrated in two of the control cases while the remaining 40 controls were completely negative. It is of interest in this respect that the host cell genome may contain many DNA copies of the homologous endogenous virus but much fewer copies of exogenous oncornaviruses [23]. This seems to be a basic difference between endogenous and exogenous oncornaviruses. Furthermore, endogenous oncornavirus p30 antigen has been demonstrated in healthy primate cells [24], and it was shown that the presence of homologous endogenous virus may make cells resistant to exogenous oncornaviruses [25]. It may therefore be supposed that endogenous oncornaviruses, which are hardly, if at all, able to transform cells, might prevent exogenous viruses from being activated. It may also be assumed that the appearance of endogenous oncornavirus antigens may indicate a breakthrough of this defence mechanism and the onset of an enhanced tendency to malignization.

The present results may be of interest mainly from the prognostical point of view. Further investigations on a greater number of patients are in progress to show whether the appearance of exogenous oncornavirus antigens in preleukaemia is a proper indicator of development into acute leukaemia. For this purpose, the virus-induced cell-surface antigens and the immune responses to the viral antigens must be investigated.

REFERENCES

1. DMOCHOWSKI, L., GREY, C. E.: *Blood* **13**, 1017 (1958).
2. HELLMANN, R., KÜFE, D., SPIEGELMAN, S.: *Proc. nat. Acad. Sci. (Wash.)* **69**, 435 (1972).
3. GALLO, R. C., MILLER, N. R., SAXINGER, W. C., GILLESPIE, D.: *Proc. nat. Acad. Sci. (Wash.)* **70**, 3219 (1973).
4. TODARO, G., GALLO, R. C.: *Nature (Lond.)* **244**, 206 (1973).
5. SHERR, C. J., TODARO, G. J.: *Proc. nat. Acad. Sci. (Wash.)* **71**, 4703 (1974).
6. STRAND, M., AUGUST, J. T.: *J. Virol.* **14**, 1584 (1974).
7. ZURCHER, C., BRINKHOF, J., BENTVELZEN, P., DE MAN, J. C. H.: *Nature (Lond.)* **254**, 457 (1975).

8. АНДЖАПАРИДЗЕ, О. Г., РАПОПОРТ, Р. И., ЮРОВСКАЯ, Г. Б.: *Вопр. вирусол.* **5**, 579 (1970).
9. GALLAGHER, R. E., GALLO, R. C.: *Science* **187**, 350 (1975).
10. NOOTER, K., AARSSSEN, A. M., BENTVELZEN, P., DE GROOT, F. G., VAN PELT, F. G.: *Nature (Lond.)* **256**, 595 (1975).
11. АНДЖАПАРИДЗЕ, О. Г., СТЕПАНОВА, Л. Г., РОЗИНА, Е. Е., ПРЯНИШНИКОВА, Л. В.: *Вопр. вирусол.* **6**, 666 (1975).
12. GREGORIADES, A., OLD, L. J.: *Virology* **37**, 189 (1969).
13. OROSZLAN, S., FISHER, C. L., STANLEY, T. B., GILDEN, R. V.: *J. gen. Virol.* **8**, 1 (1970).
14. GILDEN, R. V., OROSZLAN, S., HUEBNER, R. J.: *Nature new Biol.* **231**, 107 (1971).
15. SHERR, C. J., FEDELE, L. A., BENVENISTE, R. E., TODARO, G. J.: *J. Virol.* **15**, 1440 (1975).
16. PARKS, W. P., SCOLNICK, E. M., ROSS, J., TODARO, G. J., AARONSON, S. A.: *J. Virol.* **9**, 110 (1972).
17. STRAND, M., AUGUST, J. T.: *J. Virol.* **13**, 171 (1974).
18. BOYLE, W.: *Transplant. Proc.* **6**, 761 (1968).
19. АНДЖАПАРИДЗЕ, О. Г., ЛЮЗНЕР, А. Л., СТЕПАНОВА, Л. Г., ШУХМИНА, Н. Р., ШЕВЕЛЕВ, Б. И.: *Вопр. вирусол.* **6**, 686 (1972).
20. VIOLA, M. V., FRAZIER, M., WIERNIK, P. H., MCCREDIE, K. B., SPIEGELMAN, S.: *New Engl. J. Med.* **294**, 75 (1975).
21. GALLAGHER, R. E., SALAHUDDIN, S. Z., HALL, W. T., MCCREDIE, K. B., GALLO, R. C.: *Proc. nat. Acad. Sci. (Wash.)* **72**, 4137 (1975).
22. VOSIKA, G. J., KRIVIT, W., GERRARD, J. M., COCCIA, P. F., NESBIT, M. E., COALSON, J. J., KENNEDY, B. J.: *Proc. nat. Acad. Sci. (Wash.)* **72**, 2804 (1975).
23. BENVENISTE, R. E., TODARO, G. J.: *Nature (Lond.)* **252**, 170 (1974).
24. SHERR, C. J., BENVENISTE, R. E., TODARO, G. J.: *Proc. nat. Acad. Sci. (Wash.)* **71**, 3721 (1974).
25. TODARO, G. J., BENVENISTE, R. E., CALLAHAN, R., LIEBER, M. M., SHERR, C. J.: *Cold. Spr. Harbor Symp. quant. Biol.* **39**, 1159 (1975).

Address of the authors:

FERENC D. TÓTH, LAJOS VÁCZI, IMRE RÉDAI
Institute of Microbiology, University Medical School
H-4012 Debrecen, P. O. B. 17, Hungary

JÁNOS JAKÓ, KÁLMÁN RÁK
Second Department of Medicine
H-4012 Debrecen, P. O. B. 20, Hungary

PRELIMINARY REPORT

EXPERIMENTS CONCERNING THE SPECIFICITY AND
MECHANISM OF ACTION OF HB_sAg IN NICOTIANA
SYLVESTRIS

I. HOLLÓS, ÁGNES PÁLFI, BEATRIX GYÖRGY and KATALIN BERTA

National Institute of Hygiene, Budapest, Horticultural Research Institute, Budapest, and "László"
Central Hospital for Infectious Diseases, Budapest

(Received March 3, 1977)

Leaves of tobacco plants inoculated with vaccinia virus or with serum samples from patients with acute HB_sAg-positive hepatitis developed necrotic changes 7–14 days after inoculation. Similar alterations could be induced in certain leaves by administration of PBS. HB_sAg and vaccinia virus titres decreased to undetectable level within 16–24 hr in the inoculated leaves and neither of these agents showed diffusion to the uninoculated leaves. A significant antiviral effect of extracts from leaves inoculated with vaccinia virus could be demonstrated when extract virus mixtures were inoculated on chorioallantoic membrane of embryonated eggs. Extracts from leaves inoculated with PBS exerted but a less pronounced viral inhibition.

BANATVALA and PAYNE [1] induced necrotic changes in leaves of tobacco plants with HB_sAg-positive sera rubbed in the leaves. The leaves became wilted and stunted. They considered the effect specific, as it could be prevented with anti-HB_s and could not be induced by convalescence sera from patients with acute infectious hepatitis. Several authors have made efforts to reproduce these results. Some of them [2, 3] reported more or less success while others [4–6] failed to reproduce them. HOWARD and MCCARTHY [5] suggested that the phenomenon is non-specific, it can occasionally be induced by mechanical effects or the diluent itself.

We have treated leaves of tobacco plants (*Nicotiana glauca*) with native serum from patients with HB_sAg-positive acute hepatitis: 0.05 ml serum was inoculated into the main vein at the junction of a side-vein. Three or four leaves of each plant were inoculated. Another group of tobacco plants was treated with Lister strain vaccinia virus and a third group received PBS.

Part of the inoculated plants was observed daily for two weeks. From other plants one inoculated leaf was cut off 3, 16, 24 and 48 hr after inoculation and ground with 0.5 ml saline. The HB_sAg in the leaves was retitrated by a micro-reverse-passive haemagglutination test [7] with the Hepanosticon (Organon, Oss) reagent, whereas the vaccinia virus was determined by pock counting on the chorionic membrane of embryonated eggs pre-incubated for 11 or 12 days [8].

Table I shows that all the leaves inoculated either with HB_sAg or vaccinia virus showed at least local necrosis at the site of inoculation. Seven of the 12

Table I

Occurrence of necroses on tobacco-leaves inoculated with HB_sAg or vaccinia virus and the results of retitration

Inoculum	Dose/leaf	Ratio of necrotized leaves	Titre of leaf extracts harvested after hours			
			3	16	24	48
HB _s Ag positive sera	2048*	12/12	6*	—	—	—
Vaccinia virus	5 × 10 ⁶ **	12/12	10**	0.5**	—	—
PBS	0.05 ml	7/12	—	—	—	—

* Haemagglutination units

** Pock-forming units

leaves inoculated with PBS showed similar changes. Both the HB_sAg and the vaccinia virus disappeared from the leaves before the local necrosis had appeared. The non-inoculated leaves of inoculated plants contained no HB_sAg or vaccinia virus 3 hr after inoculation. Even antigens of the vaccinia virus could not be demonstrated by the immunodiffusion test at 24 hr and no HB_sAg was detected with the Hepanosticon reagent in the 16th hour.

Experiments carried out to clarify (i) why the antigen and the infectious virus disappeared so rapidly, and (ii) why the necrotic changes appeared so late after the virus had disappeared. In this way, we wished to recognize the factor causing the necrosis.

To answer the first question, the vaccinia virus model was used, assuming that if a substance active against vaccinia virus is formed in the leaves, it can be demonstrated by the chorion membrane assay [9], which is sensitive to antiviral agents. To demonstrate the inhibitor, we mixed the ground leaves clarified by centrifugation with a vaccinia virus suspension containing 50–70 p. f. u./0.1 ml. The mixture was kept at room temperature for 20 min and inoculated in pseudo-air sacs prepared in 6 to 10 embryonated eggs. Results are summarized in Table II.

Table II

Inhibitory effect of tobacco-leaf extract on vaccinia virus multiplication

Extracts from the leaves inoculated with	Percentage inhibition after inoculation (hours)			
	3	16	24	48
Vaccinia virus	48	92	84	40
PBS	35	70	50	29
—	30	36	40	35

Some 30–40% reduction in pock count was caused by the suspensions of uninoculated leaves. The suspensions of leaves inoculated with PBS consistently caused a more pronounced reduction. The inhibitory effect of the leaves inoculated with vaccinia virus was the most striking and showed a well-reproducible maximum at 16 or 24 hr after inoculation. The similar tendency in the PBS-inoculated leaves may be regarded as non-specific.

When titrating an inhibitory substance on the chorionic membrane we do not regard an effect significant unless it exceeds 50% reduction in pock count. In the case of control leaves, the slight inhibition might be attributed to traces of nicotine, which might act *via* the embryonated egg. To exclude the nicotine effect, we performed experiments on leaves of *Phaseolus vulgaris*. The results were consistent with those obtained with *Nicotiana* (Table II) except that the suspensions of untreated control elicited practically no pock reduction.

The curves representing the inhibitor contents of the leaves inoculated with PBS and of those inoculated with vaccinia virus agreed well with the corresponding curves calculated from the experiments on *Nicotiana* even in their time course.

Pock-reduction experiments were not carried out with HB_sAg-positive sera because the donors of the samples (adult male patients) had been vaccinated against smallpox several times, so that the antibodies in their sera would have disturbed the reaction.

We suppose that the virus-inhibitor demonstrated by us corresponds to the "plant antiviral factor" described by SELA and APPLEBAUM [10] and LOEBENSTEIN and ROSS [11], a factor thought to be an interferon-like substance acting against plant viruses [10]. It is formed in virus-infected plant cells or those inoculated with some bacteria [12]. Chemically, it is a protein, bound perhaps to RNA [13]. Its synthesis is inhibited by actinomycin D [14].

Our results indicate that in the embryonated egg system an antiviral factor of plant origin may inhibit a vertebrate virus. The antiviral factor has not yet been isolated and its mechanism is unknown. It is also unknown whether the same substance, or some simple physical or chemical noxa, are responsible for the necrotic changes.

REFERENCES

1. BANATVALA, J. E., PAYNE, C.: *Lancet* **1**, 1359 (1973).
2. ESANU, V., BABES, V. T.: *Rev. roum. Virol.* **25**, 181 (1974).
3. BUDAI, J., GYÖRGY, B., SZÉCSEY, GY., BERTA, K.: *Acta microbiol. Acad. Sci. hung.* **24**, 111 (1977).
4. BEASLEY, R. P., SU, H.: *Lancet* **2**, 1203 (1973).
5. HOWARD, C. R., MCCARTHY, D. A.: *Lancet* **2**, 219 (1974).
6. DHALIWAL, A. S., RUBINSTEIN, H. M., DIETZ, A. A.: *Lancet* **1**, 1248 (1975).
7. HOLLÓS, I., PÁLFI, Á., KŐSZECHY, Zs., NOVÁK, E.: *Orv. Hetil.* **116**, 2996 (1975).

8. HOLLÓS, I., LOVAS, B.: *Acta microbiol. Acad. Sci. hung.* **15**, 269 (1968).
9. HOLLÓS, I., NYERGES, G.: *Acta microbiol. Acad. Sci. hung.* **15**, 213 (1968).
10. SELA, I., APPLEBAUM, S. W.: *Virology* **17**, 543 (1962).
11. LOEBENSTEIN, G., ROSS, A. F.: *Virology* **20**, 507 (1963).
12. KLEMENT, Z., KIRÁLY, Z., POZSÁR, B.: *Acta phytopath. Acad. Sci. hung.* **1**, 11 (1966).
13. MICZYNSKI, K. A., MACNEILL, B. H.: *J. Phytopath.* **85**, 251 (1976).
14. GIANINAZI, S., MARTIN, C.: *J. Phytopath.* **83**, 23 (1975).

Address of the authors:

IVÁN HOLLÓS, ÁGNES PÁLFI
National Institute of Hygiene
H-1966 Budapest, P. O. B. 64, Hungary

BEATRIX GYÖRGY
Horticultural Research Institute
Nagytétényi út 198, H-1223 Budapest, Hungary

KATALIN BERTA
"László" Central Hospital for Infectious Diseases
Gyáli út 5-7, H-1097 Budapest, Hungary

BOOKS RECEIVED

ROSE, N. R., FRIEDMAN, H. (eds): *Manual of Clinical Immunology*. American Society for Microbiology. Washington, D. C. 1976. pp. XXV + 932. Price U. S. \$ 16.00 (flexible binding), 20.00 (cloth binding).

HOBSON, P. N.: *The Microflora of the Rumen*. In COOK, J. G. (ed.): *Patterns of Progress (Microbiology) Series*. Meadowfield Press Ltd., Shildon, Co. Durham, England. pp. 49. Price £ 1.90, U. S. \$ 5.70.

KIMBERLIN, R. H.: *Scrapie in the Mouse*. In COOK, J. G. (ed.): *Patterns of Progress (Microbiology) Series*. Meadowfield Press Ltd., Shildon, Co. Durham, England. pp. 77. Price £ 2.80, U. S. \$ 8.40.

STRANGE, R. E.: *Microbial Response to Mild Stress*. In COOK, J. G. (ed.): *Patterns of Progress (Microbiology) Series*. Meadowfield Press Ltd., Shildon, Co. Durham, England. pp. 83. Price £ 2.80, U. S. \$ 8.40.

VELDKAMP, H.: *Continuous Culture in Microbial Physiology and Ecology*. In COOK, J. G. (ed.): *Patterns of Progress (Microbiology) Series*. Meadowfield Press Ltd., Shildon, Co. Durham, England. pp. 68. Price U. S. \$ 8.40.

BRIDGES, B. A.: *Bacterial Reaction to Radiation*. In COOK, J. G. (ed.): *Patterns of Progress (Microbiology) Series*. Meadowfield Press Ltd., Shildon, Co. Durham, England. pp. 72. Price not stated.

SCHLESSINGER, D. (ed.): *Microbiology — 1976*. American Society for Microbiology. Washington, D. C. 1976. pp. vii + 587. Price U. S. \$ 22.00.

RATLEDGE, C.: *The Mycobacteria*. In COOK, J. G. (ed.): *Patterns of Progress (Microbiology) Series*. Meadowfield Press Ltd., Shildon, Co. Durham, England. pp. 130. Price U. S. \$ 9.60.

HARRISON, D. E. F.: *The Oxygen Metabolism of Microorganisms*. In COOK, J. G. (ed.): *Patterns of Progress (Microbiology) Series*. Meadowfield Press Ltd., Shildon, Co. Durham, England. pp. 69. Price U. S. \$ 8.40.

PRIMROSE, S. B.: *Bacterial Transduction*. In COOK, J. G. (ed.): *Patterns of Progress (Microbiology) Series*. Meadowfield Press Ltd., Shildon, Co. Durham, England. pp. 43. Price U. S. \$ 5.70.

GORDON SMITH, C. E.: *Epidemiology and Infections*. In COOK, J. G. (ed.): *Patterns of Progress (Microbiology) Series*. Meadowfield Press Ltd., Shildon, Co. Durham, England. pp. 50. Price U. S. \$ 5.70.

INDEX

<i>Kuryłowicz, W.</i> : The Site of Antibiotic Accumulation in Streptomycetes and <i>Penicillium chrysogenum</i>	263
<i>Nyerges, G., Jaszovszky, I., Pintér, A., Hompó, Zs.</i> : Investigations into the Encephalitogenic Activity of the Hempt Vaccine in Guinea Pigs	273
<i>Khavkin, T. N., Freidlin, I. S., Sakharova, I. Ya., Ioffe, V. A.</i> : Vital Fluorescence Microscopy of Lysosomes in Cultured Mouse Peritoneal Macrophages During their Interactions with Microorganisms and Active Substances. I. Vital Staining and Fluorescence Microscopy of Macrophages	281
<i>Freidlin, I. S., Khavkin, T. N., Artemenko, N. K., Sakharova, I. Ya.</i> : Vital Fluorescence Microscopy of Lysosomes in Cultured Mouse Peritoneal Macrophages During their Interactions with Microorganisms and Active Substances. II. Interactions of Macrophages with a Non-Pathogenic Strain of <i>Escherichia coli</i>	293
<i>Budai, J., György, B., Szécsey, Gy., Berta, K.</i> : Effect on Plants of Sera from Patients with Hepatitis	303
<i>Simon, M.</i> : Exclusion of False Positive Reactions Due to IgG-Binding Fc-Receptor(s) in Herpesvirus Serology	307
<i>Nagy, G., Mezey, I., Molnár, E.</i> : Simple Ion Exchange Batch Technique for Detection of IgM Antibodies Against Rubella and Flavivirus Antigens	317
<i>Tálas, M., Zakharova, L. G., Stöger, I., Altstein, A. D., Piker, E. G.</i> : Quantitative Analysis of MC29 Virus in Plasma of Hepatoma-Bearing Turkey Poults by Determination of Reverse Transcriptase Activity and Radioimmuno-Assay	323
<i>D. Tóth, F., Jakó, J., Vácz, L., Rák, K., Rédei, I.</i> : Immunological Demonstration of Oncornavirus Genome in Leucocytes in Acute and Chronic Myelogenous Leukaemia and Preleukaemia	331
<i>Hollós, I., Pálfi, Á., György, B., Berta, K.</i> : Preliminary Report. Experiments Concerning the Specificity and Mechanism of Action of HB _s Ag in <i>Nicotiana glauca</i>	341
Books Received	345

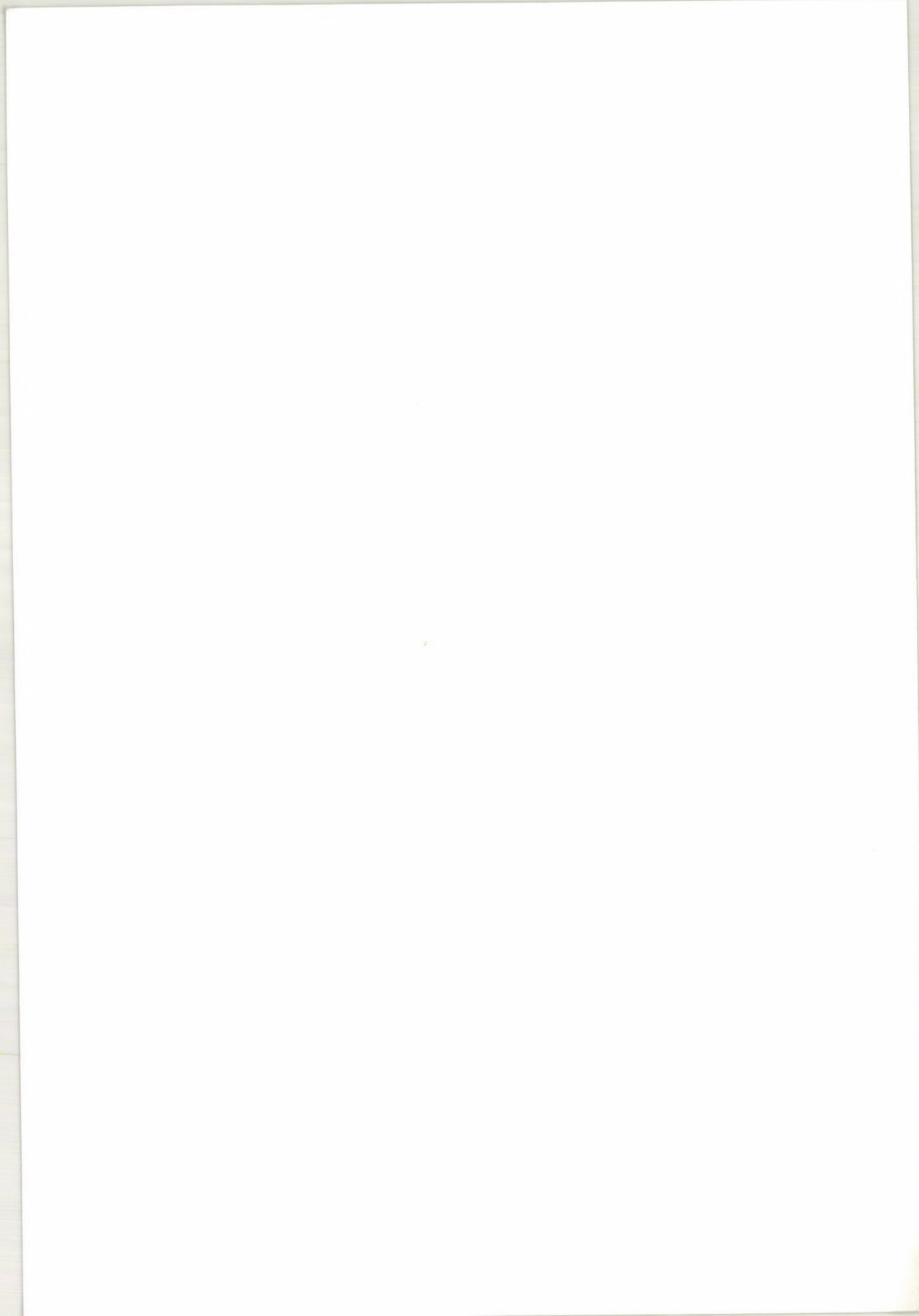
Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója.

Műszaki szerkesztő: Zacsik Annamária

A kézirat nyomdába érkezett: 1977. VIII. 26. — Terjedelem: 7,70 (A/5) ív, 32 ábra, (6 színes)

78.4063 Akadémiai Nyomda, Budapest — Felelős vezető: Bernát György



Die *Acta Microbiologica* veröffentlichen Abhandlungen aus dem Bereiche der Mikrobiologie in deutscher, englischer, französischer und russischer Sprache.

Die *Acta Microbiologica* erscheinen in Heften wechselnden Umfanges. Mehrere Hefte bilden einen Band.

Die zur Veröffentlichung bestimmten Manuskripte sind an folgende Adresse zu senden:
Acta Microbiologica, Staatliches Institut für Hygiene, H-1966 Budapest, P. O. B. 64, Ungarn

An die gleiche Anschrift ist auch jede für die Redaktion bestimmte Korrespondenz zu richten. Abonnementspreis pro Band: \$ 32.00.

Bestellbar bei dem Außenhandels-Unternehmen «Kultúra» (H-1389 Budapest 62, P. O. B. 149, Bankkonto Nr. 218-10990) oder bei seinen Auslandsvertretungen und Kommissionären.

Les *Acta Microbiologica* paraissent en français, allemand, anglais et russe et publient des travaux du domaine de la microbiologie.

Les *Acta Microbiologica* sont publiés sous forme des fascicules qui seront réunis en volumes.

On est prié d'envoyer les manuscrits destinés à la rédaction à l'adresse suivante:

Acta Microbiologica, Institut National d'Hygiène Publique, H-1966 Budapest, P. O. B. 64, Hongrie

Toute correspondance avec la rédaction doit être envoyée à cette même adresse.

Le prix de l'abonnement est de \$ 32.00 par volume.

On peut s'abonner à l'Entreprise pour le Commerce Extérieur «Kultúra» (H-1389 Budapest 62, P. O. B. 149, Compte courant No. 218-10990) ou à l'étranger chez tous les représentants ou dépositaires.

«*Acta Microbiologica*» публикуют трактаты из области микробиологии на русском, немецком, английском и французском языках.

«*Acta Microbiologica*» выходят отдельными выпусками разного объема. Несколько выпусков составляют один том.

Предназначенные для публикации рукописи следует направлять по адресу:

Acta Microbiologica, Институт Здравоохранения, H-1966 Budapest, P. O. B. 64, Венгрия

По этому же адресу направлять всякую корреспонденцию для редакции.

Подписания цена — \$ 32.00 за том.

Заказы принимает предприятие по внешней торговле «Kultúra» (H-1389 Budapest 62, P. O. B. 149, Текущий счет № 218-10990), или его заграничные представительства и уполномоченные.

Reviews of the Hungarian Academy of Sciences are obtainable
at the following addresses:

AUSTRALIA

C.B.D. LIBRARY AND SUBSCRIPTION SERVICE,
Box 4886, G.P.O., Sydney N.S.W. 2001
COSMOS BOOKSHOP, 145 Ackland Street, St.
Kilda (Melbourne), Aictoria 3182

AUSTRIA

GLOBUS, Höchstädtplatz 3, 1200 Wien XX

BELGIUM

OFFICE INTERNATIONAL DE LIBRAIRIE, 30
Avenue Marnix, 1050 Bruxelles
LIBRAIRIE DU MONDE ENTIER, 162 Rue du
Midi, 1000 Bruxelles

BULGARIA

HEMUS, Bulvar Ruszki 6, Sofia

CANADA

PANNONIA BOOKS, P.O. Box 1017, Postal Sta-
tion "B", Toronto, Ontario M5T 2T8

CHINA

CNPICOR, Periodical Department, P.O. Box 50,
Peking

CZECHOSLOVAKIA

MAD'ARSKÁ KULTURA, Národní třída 22,
115 66 Praha

PNS DOVOZ TISKU, Vinohradská 46, Praha

PNS DOVOZ TLAČE, Bratislava 2

DENMARK

EJNAR MUNKSGAARD, Norregade 6, 1165
Copenhagen

FINLAND

AKATEEMINEN KIRJAKAUPPA, P.O. Box 128,
SF-00101 Helsinki 10

FRANCE

EUROPERIODIQUES S. A., 41 Avenue de Ver-
sailles, 78170 La Celle St. Cloud

LIBRAIRIE LAVOISIER, 11 rue Lavoisier, 75008
Paris

OFFICE INTERNATIONAL DE DOCUMENTA-
TION ET LIBRAIRIE, 48 rue Gay-Lussac, 75240
Paris Cedex 05

GERMAN DEMOCRATIC REPUBLIC

HAUS DER UNGARISCHEN KULTUR, Karl-
Liebknecht-Strasse 9, DDR-102 Berlin

DEUTSCHE POST ZEITUNGSVERTRIEBSAMT,
Strasse der Pariser Kommüne 3—4, DDR-104 Berlin

GERMAN FEDERAL REPUBLIC

KUNST UND WISSEN ERICH BIEBER, Postfach
46, 7000 Stuttgart 1

GREAT BRITAIN

BLACKWELL'S PERIODICALS DIVISION, Hythe
Bridge Street, Oxford OX1 2ET

BUMPUS, HALDANE AND MAXWELL LTD.,
Cowper Works, Olney, Bucks MK46 4BN

COLLET'S HOLDINGS LTD., Denington Estate,
Wellingborough, Northants NN8 2QT

WM. DAWSON AND SONS LTD., Cannon House,
Folkestone, Kent CT19 5EE

H. K. LEWIS AND CO., 136 Gower Street, London
WC1E 6BS

GREECE

KOSTARAKIS BROTHERS, International Book-
sellers, 2 Hippokratous Street, Athens-143

HOLLAND

MEULENHOF-BRUNA B.V., Beulingstraat 2,
Amsterdam

MARTINUS NIJHOFF B.V., Lange Voorhout
9—11, Den Haag

SWETS SUBSCRIPTION SERVICE, 373b Heere-
weg, Lisse

INDIA

ALLIED PUBLISHING PRIVATE LTD., 14/13
Asaf Ali Road, New Delhi 110001

150 B-6 Mount Road, Madras 600002

INTERNATIONAL BOOD HOUSE PVT. LTD.,
Madame Cama Road, Bombay 400039

THE STATE TRADING CORPORATION OF
INDIA LTS., Books Import Division, Chandralok,
36 Janpath, New Delhi 110001

ITALY

EUGENIO CARLUCCI, P.O. Box 252, 70100 Bari
INTERSCIENTIA, Via Mazzè 28, 10149 Torino

LIBRERIA COMMISSIONARIA SANSONI, Via
Lamarmora 45, 50121 Firenze

SANTO VANASIA, Via M. Macchi 58, 20124
Milano

D. E. A., Via Lima 28, 00198 Roma

JAPAN

KINOKUNIYA BOOK-STORE CO. LTD., 17-7
Shinjuku-ku 3 chome, Shinjuku-ku, Tokyo 160-91

MARUZEN COMPANY LTD., Book Department,
P.O. Box 5056 Tokyo International, Tokyo 100-91

NAUKA LTD., IMPORT DEPARTMENT, 2-30-19
Minami Ikebukuro, Toshima-ku, Tokyo 171

KOREA

CHULPANMUL, Phenjan

NORWAY

TANUM-CAMMERMEYER, Karl Johansgatan
41—43, 1000 Oslo

POLAND

WEGIERSKI INSTYTUT KULTURY, Marszał-
kowska 80, Warszawa

CKP I W ul. Towarowa 28 00-958 Warsaw

ROUMANIA

D. E. P., București

ROMLIBRI, Str. Biserica Amzei 7, București

SOVIET UNION

SOJUZPETCHATJ — IMPORT, Moscow

and the post offices in each town

MEZHDUNARODNAYA KNIGA, Moscow G-200

SPAIN

DÍAZ DE SANTOS, Lagasca 95, Madrid 6

SWEDEN

ALMQVIST AND WIKSELL, Gamla Brogatan 26,
101 20 Stockholm

GUMPERTS UNIVERSITETSBOKHANDEL AB,
Box 436, 401 25 Göteborg 1

SWITZERLAND

KARGER LIBRI AG, Petersgraben 31, 4011 Basel

USA

EBSCO SUBSCRIPTION SERVICES, P.O. Box
1934, Birmingham, Alabama 65201

F. W. FAXON COMPANY, INC., 15 Southwest
Park, Westwood, Mass. 02090

THE MOORE-COTTRELL SUBSCRIPTION

AGENCIES, North Cohocton, N. Y. 14838

READ-MORE PUBLICATIONS, INC., 140 Cedar
Street, New York, N. Y. 10003

STECHERT-MACMILLAN, INC., 7250 Westfield
Avenue, Pennsauken N. J. 08110

VIETNAM

XUNHASABA, 32, Hai Ba Trung, Hanoi

YUGOSLAVIA

JUGOSLAVENSKA KNJIGA, Terazije 27, Beograd
FORUM, Vojvode Mišića 1, 21000 Novi Sad